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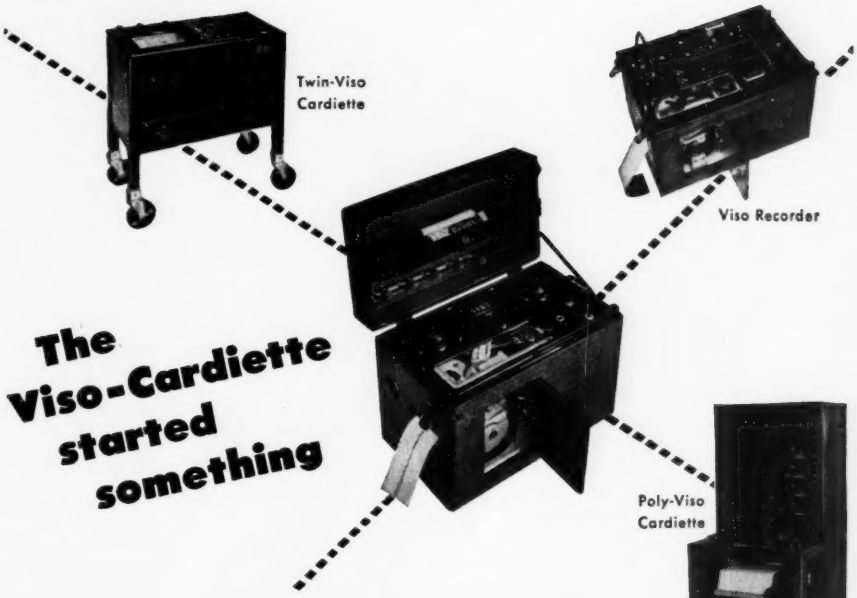

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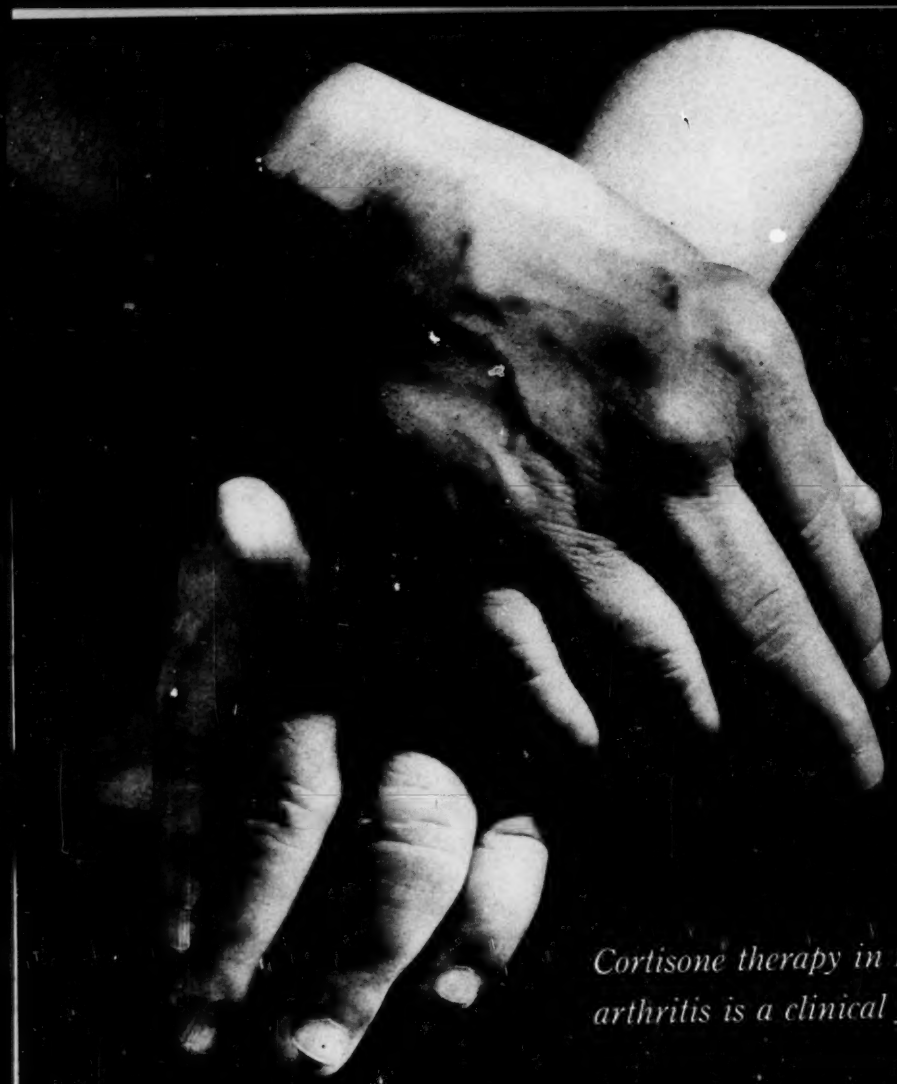

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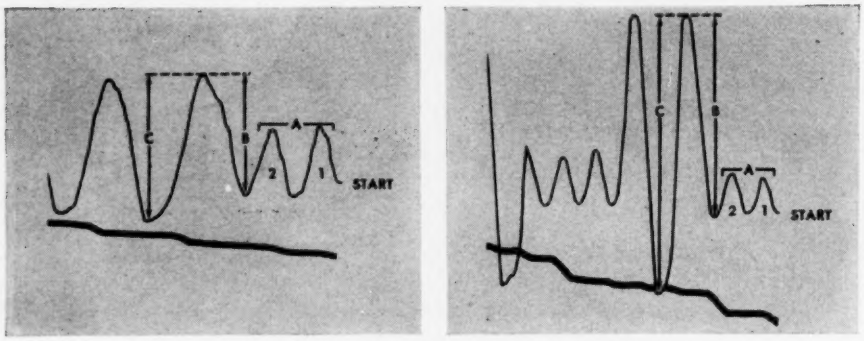

A. *Tidal breathing* B. *Complemental air* C. *Vital capacity*

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Schwartz, E., *J.A.M.A.* **147**: 1734–1737. Dec. 29, 1951.

Literature available

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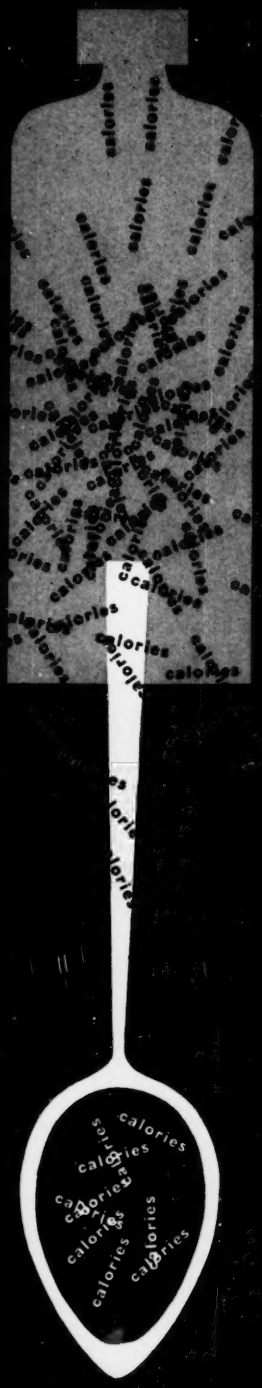

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INDICATIONS

The drug is of value in essential hypertension with fixed levels and early malignant hypertension, its effectiveness often being more marked in severe (although not terminal) phases of these disorders. It is most effective in hypertension persisting or recurring after sympathectomy.

Preliminary studies indicate worthwhile results in toxemias of pregnancy and in acute glomerulonephritis. When renal damage is advanced, as in chronic renal hypertension and chronic glomerulonephritis, the value of the drug is considerably less and it may be hazardous if not used with extreme caution.

MANNER OF USE

It is essential that the physician undertaking the prescribing or administering of Apresoline realize that the dosage requires individualization. In general, the response of each patient should be a guide to appropriate adjustment and spacing of doses.

The Ambulatory Patient

Therapy should be initiated with the *oral* form. Careful and detailed instructions should be given so that the patient will know what subjective response to expect. Within 12 to 24 hours after the first dose, headache and palpitation may occur. Often, these symptoms will disappear spontaneously with no change in dosage within 7 to 10 days, or can be ameliorated with adjunctive therapy.

Patients should be observed at frequent intervals by the physician, not only to check on the status of the blood pressure and other physiologic responses, but also to determine whether dosage change is warranted.

The initial dose of Apresoline for the average ambulatory patient with a moderate to severe degree of hypertension should be not more than 25 mg. This should be given after the morning meal and repeated after lunch, after dinner, and at bedtime. The *total* daily dose (100 mg.) is continued for one week unless side effects justify discontinuing medication. During the second week, the dose may be increased to 50 mg. four times daily (after each meal and at bedtime). This new total daily dose (200 mg.) may be continued throughout the second week.

Provided side effects are absent or minimal and the patient's blood pressure should be further reduced to a more desirable level, the single dose of Apresoline may be raised to 75 mg. four times daily for the third week, and 100 mg. four times daily for the fourth week.

Many patients obtain maximal benefit from 100 mg. of Apresoline four times daily. Some individuals are best stabilized with as little as 100 mg. daily in divided doses, while others require as much as 600 mg. daily in four equal doses.

A/ 10553M

The Hospitalized Patient

With the more severe types of hypertensive disease, 25 mg. of Apresoline may be administered *orally* every 6 hours for the first 24 hours. Thereafter, the individual doses, still at 4-6 hour intervals, are increased by 25 mg. daily until the desired effect is obtained. The average *daily* dose is 400 mg. with a range from 100 mg. to approximately 600 mg.

It has been recommended that the blood pressure be taken just prior to and one hour after each dose to determine optimal hypotensive responses and thereby, the optimal daily dose.

The *parenteral* form of Apresoline is to be used only for coma or other conditions in which the preparation cannot be taken orally. The usual dose is 20 to 40 mg. given intravenously or intramuscularly, but (especially in patients having marked renal damage) a smaller initial dosage may be indicated. Dosage increment for parenteral administration must be adjusted with even greater care than that for oral administration. The patient's blood pressure should be checked frequently after such injections, for in some cases there may be a rather precipitous drop. The pressure may begin to decline within 2 or 3 minutes after injection, the average maximal fall occurs at 25 minutes (range 10-80 minutes). If there is increased intracranial pressure, lowering the blood pressure further may result in marked cerebral ischemia. Because of this possibility, withdrawal of a suitable amount of spinal fluid may be considered. In most instances it will be possible to shift to oral therapy with Apresoline within 24 to 48 hours.

In cases of *preeclampsia or eclampsia* the blood pressure may fall dramatically to normal within 5 to 10 minutes after the intravenous injection of 20 to 40 mg. of Apresoline. The pressure has remained within normal limits for periods of 6 to 25 hours in some cases. If pressure rises again, additional parenteral doses of Apresoline may be administered.

SIDE EFFECTS

Considerable difficulty can be obviated and treatment facilitated by judiciously forewarning the patient of possible, usually transitory, side effects. This is particularly true in the case of postural hypotension and its associated effects of palpitation, headache, and nausea. In addition, certain side effects are necessarily associated with reduction of the blood pressure to more normal levels—the very purpose for giving Apresoline. In the case of postural hypotension, the patient should be told to rise slowly from a recumbent position, etc.

The cooperation of the patient will be optimal when he is taken into the physician's confidence and understands that any unpleasant effects will probably be only temporary and, if possible, should be endured to obtain the desired beneficial results. Judicious forewarning need not excite apprehension and may actually be a factor in obtaining a satisfactory effect.

The most frequent side effects include mild to severe headache, postural hypotension, tachycardia, palpitation, dizziness, weakness, nausea and vomiting. Somewhat less common are: numbness and tingling of the extremities, flushing, varying degrees of nasal congestion, lacrimation and conjunctival inflammation. A few cases of skin rash have been reported, indicating an individual sensitivity.

As Apresoline is continued, these untoward effects generally subside. If nausea or vomiting become troublesome, antacids may provide considerable relief.

Less Frequent Side Effects

Dyspnea and anginal symptoms have occasionally been reported in patients with coronary disease. The drug should be used with caution in the presence of coronary disease. Notably, however, in *some* patients having coronary disease, Apresoline has relieved anginal symptoms.

When Apresoline is given to some sympathectomized patients, they may complain of an excessively frequent urge to defecate.

If the dosage of Apresoline is increased too rapidly, especially in patients with more severe forms of hypertension and uremia, certain cerebral symptoms may accompany the fall in blood pressure. Such symptoms have been reported to range from mild anxiety or depression to acute anxiety states or severe depression and coma.

Headache

Approximately 40 per cent of patients receiving Apresoline experience mild to severe headache during the first days of treatment. Generally, it seems to be centered in the occipital region, and has been described as "pounding and grinding." The headache may not be due to any direct action of the drug, but rather to an indirect result of the lowered blood pressure or even an antihistaminase action, preventing the normal destruction of histamine. Actually, the symptoms of the same type of headache can be induced by histamine. Symptoms can frequently be relieved by the use of antihistamine drugs.

It has also been suggested that some of the headache may be due to a general vasodilatation of certain cerebral or centrally located vessels, as in congestive migraine. Therefore, a number of clinicians have found that ergotamine tartrate, "in extremely small amounts," provides satisfactory control.

ISSUE

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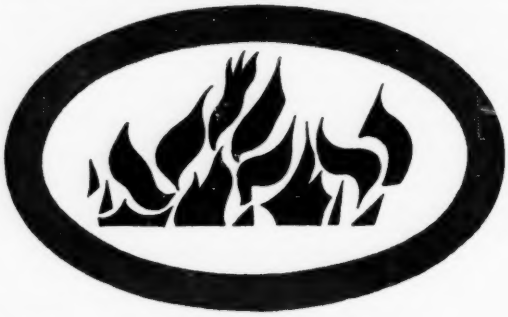

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LIPOPROTEIN PATTERNS OF SERUM OBTAINED BY ZONE ELECTROPHORESIS

By HENRY G. KUNKEL AND ROBERT J. SLATER

(From the Hospital of The Rockefeller Institute for Medical Research, New York, N. Y.)

(Submitted for publication February 25, 1952; accepted April 28, 1952)

For a number of years the lipoproteins of serum have been classified as alpha and beta types according to their electrophoretic mobility. Despite this designation very few studies of their electrophoretic properties have been reported. This is chiefly due to the fact that it is very difficult to determine exactly where the lipoproteins migrate when patterns of whole serum are obtained by the classical Tiselius method. Isolation of the lipoproteins can be achieved by chemical (1, 2) and ultracentrifugal methods (3, 4). However, even if turbidity and solubility problems in the electrophoretic separation of the isolated lipoproteins are overcome, some question remains as to whether the mobilities of these delicate complexes are the same in the isolated state as they are in whole serum. To circumvent these difficulties and to supplement the information obtained from the ultracentrifuge by Gofman, Lindgren and Elliott (3), Green, Lewis and Page (4), and Turner and associates (5) and that obtained from chemical fractionation by Lever and associates (2) and Russ, Eder and Barr (6, 7), the new technique of zone electrophoresis was applied to this problem. This method has the advantage that the electrophoretic components can be isolated directly so that lipid analyses can be carried out. The results obtained in the present study indicate that lipoprotein patterns can be obtained on whole serum and can be correlated in terms of relative electrophoretic mobility with the other proteins of serum.

MATERIAL AND METHODS

Sera were obtained from normal individuals and patients in the fasting state unless otherwise indicated. Analyses were carried out within three days after the removal of the serum except in the case of hypothyroidism where the serum had been stored at 4°C for 11 months without the development of significant sediment or lipid material at the surface. The patients with primary biliary cirrhosis were part of a group discussed previously (8). Total lipid, cholesterol and phospholipid analyses were carried out on the same alcohol-ether extract of serum or isolated protein fraction. The methods used were essentially those described in a previous report (8). Phosphorus was converted to phospholipid with the factor 25. In certain specifically designated cases cholesterol analyses on the multiple protein fractions were carried out by a simplified technique (9) where the color of the Liebermann-Burchard reaction was obtained directly for the mixture of free and esterified cholesterol in chloroform. Although this method was carefully standardized with the Sperry-Schoenheimer procedure, it was not as accurate as the latter because of variation in the esters which gave a slightly more intense color. It was used for the curves illustrated in Figures 3, 4 and 5. For Figures 1 and 2 and Table I the conventional Sperry-Schoenheimer technique was used.

The method of zone electrophoresis was similar to that described previously (10). Filter paper and potato starch were used as the two types of supporting media in the present study. For the filter paper experiment, three to nine sheets of Whatman 3 MM paper 14 inches in length and 4 inches in width were superimposed on each other. A rectangular segment was cut out of the block of filter paper and the segment and block immersed in buffer solution. The wet paper was then dried to a barely moist state on another sheet of thick filter paper and the blocks placed on a silicone treated glass plate. Serum (2–5 cc.) was then applied to the cut segment of paper and this was replaced in its original position in the block of filter paper. Another glass plate was placed on top of the paper and clamps applied. Silicone grease was spread along the edge of the paper block between the plates to prevent evaporation at the sides. After placing the ends of the plates on the lips of perspex vessels containing buffer in contact with electrodes in a cold room, a current of 25–60 m.a. was applied through a variable voltage source of 100–500 volts. The separation was carried out over a period of approximately 24 hours. The degree of separation could be followed by placing a light underneath the lower glass plate and observing the distance of migration of albumin which was readily visible at this high concentration of serum through the yellow bilirubin that was bound to it. Two other natural pigment bands in the β globulin region also aided in determining the degree of separation. At the conclusion of the experiment after removing the glass plates the upper sheet was stained with brom phenol blue, cut into ½ inch segments and the dye eluted to obtain a serum protein pattern (10). The lower block of paper sheets was also cut up into ½ inch segments with special shears and placed in 20 cc. hot alcohol-ether (3 : 1)

solution. The lipids were extracted over a ten hour period, the paper removed after pressing out the solvent, and the tubes centrifuged to remove precipitated protein or paper fragments. Aliquots of the clear alcohol-ether extract were then taken for cholesterol, phospholipid or total lipid analysis. The recovery of lipid from the segments was less than 100% because of removal of alcohol-ether with the paper. However, this error was relatively constant for each tube and was minimized by the excess of solvent.

Barbital buffer at pH 8.6, μ 0.1 was usually employed for the separation. Some barbital was extracted with the alcohol-ether but this did not interfere with the cholesterol or phospholipid analyses. However, total lipid carbon could not be determined because of barbital carbon. Barbital also interfered with nitrogen analyses on the fractions. Phosphate buffers were used where total lipid analyses were carried out. However, phospholipid analyses were difficult in this buffer and could only be carried out after washing the dried alcohol-ether residue with acid water to remove buffer phosphorus.

In the experiments where potato starch (Amend) was used as the supporting medium, a block of barely moist starch was used between glass plates in the same manner as filter paper. This was prepared by pouring a starch buffer mixture into a mold lined with wax paper. The starch was brought to a solid form by removing excess buffer with porous filter paper applied at the sides of the mold. The procedure will be described in detail elsewhere (11). The wax paper prevented sticking of the starch to the glass plates and could be peeled from the starch block readily at the conclusion of the experiment. Serum mixed with starch was placed in a rectangular space cut out of the starch block just as in the case of the filter paper block, or was applied directly with a pipette which penetrated into the starch. Contact at the electrode vessels was made with plastic sponges that pressed against the ends of the starch block. At the conclusion of the separation, the starch block was cut into segments with a thin spatula and the segments placed in test tubes. Saline was added to each tube (1 cc.) and after stirring the starch and allowing it to settle, 0.05 cc. of supernate was removed from each tube. This was used to obtain a protein curve of the serum segments. The modified Folin-Ciocalteu procedure (10, 12) was found most suitable for this purpose. The starch did not contain material which interfered with this reaction. Ninhydrin and ultraviolet absorption curves could also be obtained but they required considerable washing of the starch to remove interfering material. Hot alcohol-ether was then added to the saline starch mixture or preferably to partially dried starch segments and the lipids extracted. Aliquots were taken for cholesterol and phospholipid analyses. Total lipid determinations were difficult because of some carbon material in the starch which was difficult to remove. Good recoveries could be obtained from the starch segments by further addition of solvent on a ground glass filter. The fresh solvent then displaced that mixed with the starch by suction. However, for most experiments a single extraction was used with a constant loss of solvent for each tube. Initial difficulties in the complete extraction of the cholesterol esters were overcome by longer extraction of the starch segments with hot solvent.

In the experiments where the lipoproteins were stained directly on filter paper after separation of the serum, the dried paper was immersed in a 50% ethanol solution saturated with Sudan III, placed at 37°C for three hours and washed with running water. Two superimposed filter paper strips were run together in these experiments and one strip, stained by the conventional method, was compared with the strip where only the lipid of the lipoprotein was stained.

Adsorption of proteins to the filter paper or starch was measured by letting buffer flow through the medium without an electric current. This was done by lowering the liquid level in one electrode vessel producing a siphon-like arrangement. The protein was carried along, leaving a trail if significant adsorption occurred. No separation of the serum proteins was obtained unless current was applied.

RESULTS

Preliminary observations on the localization of the lipoproteins relative to the other protein components of serum were carried out by simply staining the filter paper with Sudan III or other similar fat soluble dyes. Normal serum showed two distinct components, an intense spot in the β globulin region and a more faintly staining spot in the albumin-α_1 region. By careful comparison of the Sudan stained strips with an identical strip stained with brom phenol blue where all of the protein peaks were visible, it could be seen that the β Sudan spot was close to the β globulin peak but usually with a slightly lower mobility. The fainter Sudan spot did not correspond to the α_1 peak but showed a slightly more rapid mobility. It lay partially under the albumin peak. Pathological sera with high lipid concentration showed many variations of the Sudan III patterns. Intense spots were visible in new positions on the pattern. Nephrotic sera usually showed a single spot moving in the region between the two main spots visible in the normal pattern. There appeared to be a general tendency for the normal lipid components to disappear and be replaced by new components, usually single, with a different mobility.

To test the validity of these observations and to determine whether the Sudan III was really staining the lipoprotein components selectively,

direct lipid analyses were made on paper segments. To do this successfully it was necessary to increase greatly the capacity of the procedure so that 2–5 cc. of serum could be separated instead of 0.01 cc. as in the simple pattern stained with Sudan III. This was found possible by superimposing a number of sheets of filter paper, forming a block between glass plates. Figure 1 illustrates the protein pattern obtained by the brom phenol blue dye elution method in segments of the paper and the cholesterol and phospholipid patterns for the same segments. For this pattern 2.5 cc. of dialyzed serum were used with barbital buffer at pH 8.6, μ 0.1 and a current of 40 m.a. over a period of 24 hours in the cold room. Two major lipid peaks are visible, one slightly behind the β globulin (designated β) and the other slightly behind the albumin but ahead of the α_1 globulin (designated α). This experiment confirms the localization of the lipoproteins observed with the Sudan staining. A third vague peak is visible extending from the origin to the β peak. This vague area was also visible in the experiments with Sudan III. It has the appearance of a trail left behind as the β component migrated from the origin. However, it is of interest that

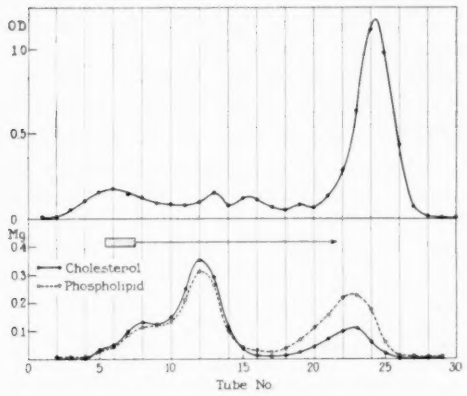

FIG. 1. CURVES SHOWING THE α AND β LIPOPROTEINS FOR A NORMAL SERUM SEPARATED BY ZONE ELECTROPHORESIS IN A FILTER PAPER MEDIUM

The upper curve represents the protein pattern obtained by the brom phenol blue elution method, with O.D. referring to dye color. The lower curves represent the cholesterol and phospholipid values obtained in the same experiment on identical segments of paper.

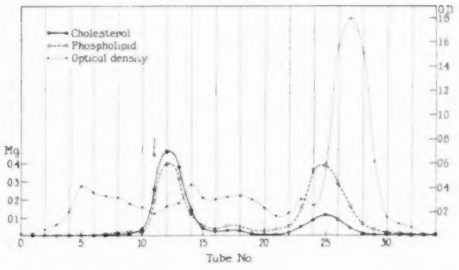

FIG. 2. CURVES SHOWING THE α AND β LIPOPROTEINS FOR A NORMAL SERUM SEPARATED BY ZONE ELECTROPHORESIS IN A STARCH MEDIUM

Protein, cholesterol and phospholipid analyses were carried out on the same starch segments. The optical density refers to the color intensity obtained with the modified Folin method of protein determination. The arrow signifies the origin.

total lipid analyses along with the cholesterol and phospholipid determinations indicated that this vague peak contained considerably more neutral fat than the β or α peaks.

To further confirm the observations on the mobility of the lipoproteins relative to the other proteins of serum and to eliminate possible trailing of the β component, a search was made for other supporting media in which to carry out the electrophoretic separation. Various materials were tried, glass beads and powders, resins, super cel and agar among others. All of these media caused considerably greater electroosmotic flow of water than did the paper (10) and the adsorption of the lipoproteins was considerable. Potato starch was finally settled on as the most satisfactory medium. No adsorption of the lipoproteins could be demonstrated. The electroosmotic flow was slightly greater than with the paper but not enough to detract from the usefulness of the starch. Lysozyme, another protein that was strongly adsorbed to filter paper in alkaline buffers, showed no detectable adsorption on the starch.

In Figure 2 the protein curve, determined by analyses with the modified Folin procedure on starch segments, is plotted along with cholesterol and phospholipid determinations for a different normal serum than that shown in Figure 1. In general the agreement was good. The β lipoprotein is in approximately the same position as in the separation on filter paper. The α lipopro-

TABLE I

Cholesterol-phospholipid and free-total cholesterol ratios in the α and β lipoprotein components of serum from five normal young males

The analyses were made on the tubes containing the highest concentration of cholesterol for each component.

α Component		β Component	
Chol/P lipid	F/T chol	Chol/P lipid	F/T chol
0.46	0.20	1.31	0.22
0.62	0.21	1.36	0.25
0.44	0.23	1.17	0.28
0.60	0.25	1.21	0.29
0.41	0.23	1.25	0.31

tein migrated slightly slower in this experiment but it still was ahead of the α_1 globulin, partially in the albumin fraction. A large number of determinations by the starch and filter paper procedures showed no essential difference in the relative positions of the α and β lipoproteins by the two methods. The β was always in the β globulin position or slightly behind it while the α was always in the region between the α_1 globulin and albumin peak. When large volumes of serum were used in the separation it was not always possible to observe the α_1 globulin peak as clearly as in Figure 1 which represented an unusually good pattern. Protein curves were obtained by nitrogen analyses, ultraviolet absorption and by the ninhydrin technique as well as by the method mentioned. Certain differences were noted; however, regardless of the method of protein estimation, the α_1 globulin peak was consistently observed along with the four other major peaks when barbital buffer (pH 8.6, μ 0.1) was used. In other buffers the α_1 globulin peak was not seen. Experiments carried out in phosphate buffer at pH 7.6, μ 0.1, showed the α lipoprotein peak to fall right in the albumin fraction with the major peak sometimes slightly ahead of the albumin peak.

The major difference between the lipoprotein curve in Figures 1 and 2 was the almost complete absence of material with a slower mobility than the β lipoprotein in the starch pattern. A slight peak is observed in Figure 2 near the origin but in other patterns on normal serum it was completely absent. Figure 2 also shows a small clearly defined component in the region between the α and β lipoprotein peaks. This was seen occasionally in both the starch and filter paper in barbital buffer at pH 8.6. In addition to the absence of the trailing material, the starch system gave better recoveries and more uniform separation of the lipoproteins than did the filter paper.

In addition to defining the relative mobilities of the lipoproteins, Figures 1 and 2 demonstrate the low cholesterol to phospholipid ratio of the α lipoprotein as compared with the β. A similar relationship was found in 25 different normal sera that were studied. The phospholipid of the α peak was usually lower than the phospholipid of the β component as in Figure 1 but an occasional serum was found to have the α phospholipid higher than the β. They are approximately equal in the normal serum of Figure 2. Determinations of cholesterol esters in the α and β components showed that the F/T ratio was consistently lower in the α fraction than in the β (Table I). The trailing component visible in the filter paper experiments which was relatively high in neutral fat showed approximately the same cholesterol-phospholipid ratio as did the β lipoprotein.

Observations with acetate and phosphate buffers in a pH range from 5 to 7.6 gave results that were in general similar to those with barbital when the ionic strength was kept at 0.1. Some separation of the α lipoprotein was sometimes seen and the β lipoprotein gave a more diffuse peak; the basic division of the lipoproteins into two main groups continued to be evident. However, if the buffer concentration was reduced to an ionic strength of 0.05, a definite subdivision of the main components was observed. In barbital buffer at

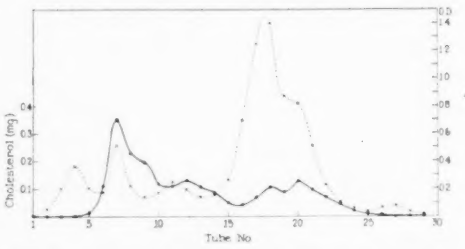

FIG. 3. FURTHER SUBDIVISION OF THE α AND β LIPOPROTEINS FOLLOWING ELECTROPHORESIS IN A STARCH MEDIUM USING PHOSPHATE BUFFER pH 6.5, μ 0.05

The cholesterol curve is shown (solid line) along with that for O.D. of protein color (dotted line). The β lipoprotein peaks are in tubes 7, 9 and 12; the α peaks in tubes 18 and 20. The origin was in tube 10.

pH 8.6, μ 0.05, the α component split up into two and sometimes as many as four fractions. The β component always separated into at least two peaks. At a lower pH, three β fractions were consistently observed. Figure 3 illustrates the subdivision of the lipoproteins into at least five components at pH 6.5, μ 0.05. The splitting of the albumin peak at this pH was an unexpected finding and is of interest in other connections. The high cholesterol peak in tube 7 was observed during the course of the experiment as a sharp orange colored band. It was peculiar in that the band was narrower than the origin and of such intensity of color that a grouping together of lipoprotein particles must have occurred, carrying pigment with them. A surprisingly high lipid concentration was always found in this band. The lipemic particles of non-fasting serum also moved in this band. At lower pH levels in acetate buffers the colored band was also visible but migrated in an intermediate position among the other lipoprotein peaks. This phenomenon probably resulted from boundary anomalies due to the high protein to buffer ratios.

Pathological sera

Lipoprotein curves were obtained for a number of pathological sera employing the filter paper procedure. The adsorption and trailing of the β lipoprotein was a greater problem, particularly with lipemic sera, than it was with the normal sera mentioned above. However, again total lipid analyses indicated that the trailing component was high in neutral fat. To avoid this problem the starch medium with barbital buffer pH 8.6, μ 0.1 was adopted for the main observations on high lipid sera. Figure 4 illustrates the lipoprotein curves obtained for three different pathological sera and a normal serum where the colored albumin band was permitted to migrate the same distance from the origin in each case. The marked variation from the normal is apparent.

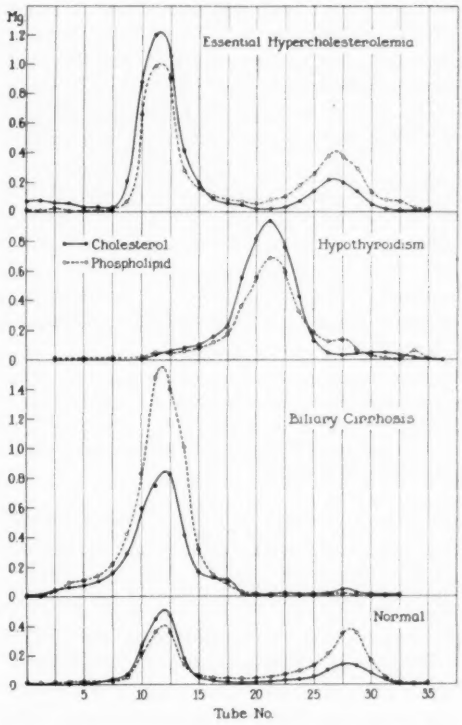

FIG. 4. LIPOPROTEIN PATTERNS FOR THREE PATHOLOGICAL SERA CONTRASTED WITH THAT OF A NORMAL SERUM EMPLOYING A SIMILAR DEGREE OF SEPARATION IN EACH CASE

The origin was in tubes 8 and 9; 2.5 cc. of each serum were used, pH 8.6, μ 0.1.

TABLE II

Results of lipid determinations on the whole sera used in Figures 4 and 5

	Total lipid	Cholesterol	Phospholipid
Normal (Fig. 4)	691	220	260
Xanthoma	1,230	380	610
Hypothyroid	1,397	452	375
Essential Hypercholesterolemia	1,532	550	522
Nephrosis A	2,741	733	411
Nephrosis B	805	282	320

Table II furnishes data on the lipid composition of these sera. The pattern for the biliary cirrhosis serum is characteristic for this disease and is similar to that observed in the serum of four other patients. A very high peak consisting predominantly of phospholipid migrated at approximately the same relative mobility as normal β lipoprotein. The α lipoprotein was extremely low. In two of the five sera it was completely absent. In one case the main peak was notched indicating partial splitting of this component. In addition to the five high lipid sera from patients

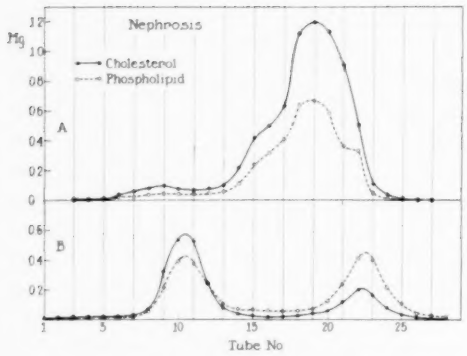

FIG. 5. LIPOPROTEIN PATTERNS OBTAINED IN A PATIENT WITH NEPHROSIS, BEFORE (A) AND AFTER A SPONTANEOUS DIURESIS (B)

The origin was in tubes 8 and 9, with a similar degree of separation in each case.

with biliary cirrhosis, two sera were examined from patients with this condition who at one time had lipid abnormalities but who despite continuation of jaundice developed levels in the low normal range. These sera showed a pattern similar to that just described with almost complete absence of the α lipoprotein peak and all the lipid in a component resembling the normal β lipoprotein in terms of relative mobility. Two sera were also obtained from patients with obstructive jaundice due to a stone in one case and carcinoma of the head of the pancreas in the other. These sera also showed a similar pattern with a disappearance of the α lipoprotein component.

The pattern from the serum of the patient with hypothyroidism also shows a low α lipoprotein peak and little or no lipid in the position of the usual β component. The one large component has an intermediate mobility. This was the only hypothyroid pattern available so that it is impossible to say that it was characteristic of this disease.

Three sera were examined from individuals with essential hypercholesterolemia; two were of the familial type. None of these individuals had any real complaints. One showed xanthelasma. The pattern in Figure 4 is typical of this group. The α lipoprotein was normal or moderately depressed while the β lipoprotein was considerably increased, slightly more for the cholesterol than for the phospholipid. No components with abnormal mobilities were observed.

Four sera were examined from children with nephrosis. Considerable variation was observed but in general one large irregular peak consisting of from one to three components was observed in the region between the α and β peaks of normal serum. The β peak was always almost completely absent. The α peak appeared markedly reduced but because of overlap by the main abnormal component it was sometimes difficult to differentiate. Figure 5A illustrates a pattern obtained in one of the patients at a time when edema, ascites and severe albuminuria were present. The patient developed a diuresis, probably spontaneously, with complete loss of retained fluid, a decrease in the albuminuria, and a gain in body weight. A second pattern obtained seven months after the first is shown in Figure 5B, with the same degree of separation. The second pattern is essentially normal.

DISCUSSION

Many of the observations mentioned above, particularly those concerning the composition of the α and β lipoproteins, have been reported previously (*2*, *6*), from experiments where the lipoproteins were isolated by the methods of Lever and associates (*2*). It is of interest that most of the results for normal serum are in approximate agreement despite the basic differences in the methods of chemical and electrophoretic separation. The normal lipoproteins certainly can be divided into the two main classes, the β type with a cholesterol phospholipid ratio of approximately 1.3 and the α type with a ratio of approximately 0.5. The finding of a consistently lower free-total cholesterol ratio in the α lipoprotein than in the β demonstrates a further difference.

Of some interest is the finding that the α lipoprotein migrates slightly ahead of the α_1 globulin component and falls partially in the albumin fraction of serum. In phosphate buffer at pH 6.5 it actually migrated partially ahead of the major albumin peak. Attempts have been made to correlate the α_1 globulin area in free electrophoresis with the concentration of α lipoprotein determined by other methods. These have not been particularly successful and perhaps the slightly greater mobility of the lipoprotein is the explanation. It

should be mentioned that in free electrophoresis experiments the lipid of the lipoproteins contributes to the refractive index increment of the proteins while in the present experiments the lipid was not estimated by either the dye elution or the modified Folin procedure. This accounts for some of the differences in the protein curves obtained by the two types of electrophoresis discussed previously (10). The slightly slower mobility of the β lipoprotein compared to the β globulin peak may be explained in this way; and, if the lipid were measured as in free electrophoresis experiments, these two peaks might fuse. A similar explanation might apply to the α_1 globulin peak although here the influence of the lipid would be less marked.

A large scale quantitative analysis of the normal fractions, such as that carried out by Russ, Eder and Barr (6) with their striking results of sex and age differences, is more practically done by the chemical methods. The results with the zone electrophoresis method certainly confirm the validity of the chemical division for such relatively normal sera. For pathological sera with large lipid alterations the results of the chemical method are more difficult to interpret and do not correlate as well with the results of zone electrophoresis. Some of the pathological sera examined in the present study, particularly those from patients with nephrosis, showed the major lipoprotein to have an intermediate mobility clearly different from the α and β components of normal serum. By the chemical fractionation procedure this abnormal component falls chiefly in the same fraction with the normal β lipoprotein. Sera from patients with biliary obstruction, intrahepatic or extrahepatic in type, were studied particularly. Of nine such sera from different patients, not one showed a significant amount of the normal α component; it was either entirely absent or present in very low concentrations. This was a general phenomenon applying to other high lipid pathological states; in no case was an increase in the α component observed. Three patients with biliary obstruction examined by the chemical procedure have been reported (7) to have an increase in the fraction showing the α component of normal serum. These results are difficult to understand; perhaps the high phospholipid content of the lipoprotein of these sera alters their solubility so that they fall into the α fraction or perhaps the high levels of bile salts are responsible. For these pathological sera the method of zone electrophoresis would appear to possess certain advantages, particularly in demonstrating new components.

Although the procedure utilizing filter paper as the stabilizing medium showed adsorption of the β lipoprotein in the path of migration, it furnished some information not provided by the starch system. The trailing material showed a high neutral fat content relative to that of the main β component. Such a selective adsorption of the larger lipoprotein molecules containing increased amounts of neutral fat might be expected and these molecules probably correspond to the higher Sf components described by Gofman, Lindgren and Elliott (3). Of particular interest is the fact that the cholesterol-phospholipid ratio of the adsorbing component was very close to that of the β lipoprotein. This, together with the fact that the high neutral fat material had the same mobility as the β component when the nonadsorbing starch medium was used, suggests a close relationship.

The low cholesterol-phospholipid ratio obtained for the β component in the sera of patients with biliary obstruction differs strikingly from the composition of the β lipoprotein in normal serum. It may be that this represents an entirely different lipoprotein that just happens to migrate with the mobility of the normal component. However, it seems more likely that this protein is related to the normal β lipoprotein with a slightly different lipid composition. Isolation of this lipoprotein in the ultracentrifuge in preliminary experiments has indicated that it has a lipid-protein ratio close to but slightly higher than the normal β lipoprotein.

SUMMARY

1. Separation of α and β lipoproteins of normal serum into two main fractions was readily possible by zone electrophoresis in a filter paper or starch medium containing barbital buffer. Lipoprotein curves were obtained and the relative mobilities of the components correlated with the mobilities of the other serum proteins.

2. The cholesterol-phospholipid ratios of the two major components showed approximate

agreement with the results obtained by other workers by chemical fractionation. A lower free-total cholesterol ratio was found in the α fraction than the β.

3. Further subdivision of the α and β lipoproteins was obtained with low ionic strength buffers in the starch medium. The α lipoproteins consistently separated into two and sometimes three fractions. The β lipoproteins separated into at least three fractions, one of which could be seen as a sharp orange band.

4. Marked variations from the normal pattern were found in pathological sera with elevated lipid concentrations. These were characterized by a general tendency of diminution or disappearance of the α lipoproteins and either an increase of the β type or the appearance of a large component with an abnormal mobility.

ACKNOWLEDGMENT

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STUDIES ON THE INITIATION OF BLOOD COAGULATION. III. THE CLOTTING PROPERTIES OF CANINE PLATELET-FREE PLASMA [1]

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The changes which take place in shed blood and thereby initiate its coagulation remain a source of considerable speculation. It is still common belief that platelets are necessary for the *initiation* of blood coagulation although a number of investigators have presented evidence to the contrary (1–8). While platelets appear to be essential for *efficient* clotting, there is good evidence that coagulation can occur in their absence. Investigations in this laboratory revealed that human platelet-free plasma, prepared without anticoagulant, regularly clotted in glass tubes at 37°C (7). However, aliquots of the same plasma clotted much more slowly or not at all in silicone-treated tubes. It was demonstrated that the rate of clotting of platelet-free plasma was directly related to the area of the glass surface to which the plasma was exposed. These studies were interpreted as providing evidence for the existence of a "plasma thromboplastin" which is activated on contact with glass surfaces and can bring about clotting in the absence of platelets (7, 8).

On the other hand Patton and his associates obtained different results with canine plasma using similar techniques (9). In their experiments canine plasma was freed of formed elements by high-speed centrifugation. This plasma when stored at *room temperature* was observed to clot imperfectly or not at all. Fibrin formation occurred with equal frequency in glass and silicone-treated tubes. These authors interpreted their results as providing no evidence for the view that plasma contains a soluble factor which can initiate clotting independent of platelet action.

The apparent differences in the clotting activity of human and canine platelet-free plasma might be attributable to a species variation. For this reason it seemed of interest to study the clotting properties of canine plasma with the techniques previously employed for human plasma. The results demonstrated that canine platelet-free plasma clotted when exposed to glass surfaces, and that the rate of clotting was related to the glass surface area. The clotting activity of canine platelet-free plasma was qualitatively similar to that of human plasma, but there were slight quantitative differences. These differences accounted for the apparent discrepancy between results previously obtained with dog and human plasma.

METHODS AND MATERIALS

Preparation of platelet-free plasma: Normal fasting dogs, weighing 7 to 21 kilograms, were anesthetized by the injection into a saphenous vein of sodium pentobarbital, 30 mg. per kilogram. Blood specimens were obtained by jugular venipuncture. Silicone [3]-treated needles, syringes, and tubes were employed. A multiple syringe technique was used in order to eliminate contamination with tissue juice. After about 10 ml. of blood had been withdrawn, the syringe was replaced by another which had been treated both with silicone and with silicone oil.[4] Thirty to 50 ml. of blood were then withdrawn, carefully transferred to silicone-treated tubes, and centrifuged at 12,000 RPM for 10 minutes in a Sorvall angle centrifuge at 2°C. The upper portion of the plasma was carefuly removed with a silicone-treated pipet and stored in silicone-treated tubes in an icebath. No anticoagulant was used. Plasma prepared in this manner in many instances contained no more than 10 platelets per cmm.

Clotting times: The clotting times of 1 ml. portions of blood or plasma were determined in scrupulously clean Pyrex test tubes (13 × 100 mm.) at 37°C. In whole blood clotting time determinations three tubes were employed,

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[2] Postdoctorate Fellow, U. S. Public Health Service.

[3] Dri-Film 9987, General Electric Co.

[4] Silicone Oil 9996–200 centistokes, General Electric Co.

the clotting time being recorded as that found in the final tube. Clotting times on normal pound dogs varied between 17 and 36 minutes, values similar to those obtained with human blood. In clotting time determinations on platelet-free plasma two tubes were employed.

Prothrombin times: The one-stage method was employed. Rabbit brain thromboplastin was prepared according to the technique of Brambel (10). In normal dogs the prothrombin times varied from seven to 11 seconds, depending on the particular lot of thromboplastin employed.

Platelet counts: Platelet counts on whole blood were performed by a routine method employing Rees-Ecker diluent. Platelet counts on centrifuged plasma were performed by a method previously described (7). In essence this consists of enumerating the total number of platelets in 0.9 cmm. of undiluted plasma in a Spencer "Bright-Line" counting chamber with a silicone-treated coverslip.

Fibrinogen determinations: The fibrinogen concentrations of plasma were determined by the method of Ratnoff and Menzie (11).

Preparation of crushed glass: The preparation of crushed glass particles has been described (8). Those particles which passed through a 50 mesh screen but were retained by a 100 mesh screen were used.

Preparation of barbital buffer: The buffer solution was composed of 7.3 grams sodium chloride, 2.76 grams barbital, 2.06 grams sodium barbital, and sufficient distilled water to bring the total volume to one liter. The mixture was heated gently without boiling to facilitate solution. The pH of this isotonic material was 7.5 ± 0.1.

RESULTS

High speed centrifugation of canine blood in silicone-treated tubes at low temperatures regularly resulted in the virtual elimination of platelets from the supernatant plasma. Direct microscopic examination of this undiluted native plasma revealed fewer than 70 platelets per cmm., and in many instances no more than five platelets per cmm. were observed. The plasma was clear and showed no evidence of clot formation when stored in silicone-treated tubes at 2°C for a period of observation of 24 hours.

A number of observations suggest that the clotting components of this native plasma were unaltered by centrifugation except for the removal of formed elements. The addition of rabbit brain thromboplastin to platelet-free plasma in silicone-treated tubes at 37°C brought about complete clot formation in less than 12 seconds. Furthermore, the prothrombin time of the platelet-free plasma was the same as that of plasma obtained from oxalated blood by low-speed centrifugation. No appreciable change in the fibrinogen concentration of the plasma occurred during the centrifugation process. In most of the experiments platelet-free plasma did not clot when stored for 24 hours in silicone-treated tubes at 37°C. When platelet-free plasma was oxalated no evidence of clot formation was observed during 24 hours storage at 37°C even in the presence of an excess of crushed glass. These observations indicate that thrombin was not formed during the preparation of the plasma.

Platelet-free plasma was prepared in 22 experiments from the blood of 15 dogs. One experiment will be described in detail:

A fasting mongrel dog weighing 13.6 kilograms was anesthetized by the intravenous injection of 410 mg. sodium pentobarbital into a saphenous vein. Blood was drawn from a jugular vein using the technique described in the section on methods and materials. The clotting time of the whole blood in three tubes was 16, 16, and 22 minutes. A portion of the blood was oxalated by the addition of 10% by volume of M/10 sodium oxalate solution, and centrifuged at low speed for approximately 10 minutes. The prothrombin time of the supernatant plasma on repeated determinations was seven, eight, seven, and eight seconds. Another portion of the dog's blood was oxalated by introducing 5 ml. of the blood into a tube containing 4 mg. of potassium oxalate and 6 mg. ammonium oxalate. This oxalated blood was also centrifuged at low speed and the supernatant plasma was found to contain fibrinogen in a concentration of 299 and 307 mg. per 100 ml. plasma in duplicate determinations.

Forty ml. of the dog's blood were placed in tubes which were coated both with silicone and silicone oil. These tubes, which were immersed in an icebath, were transferred to the refrigerated centrifuge. They were then centrifuged at 12,000 RPM for 10 minutes. The upper portion of the supernatant plasma was carefully removed with a silicone-treated pipet.

Direct microscopic observation of this undiluted plasma in a counting chamber revealed that there were six platelets per cmm. A portion of the plasma was oxalated by the addition of one part of M/10 sodium oxalate solution to five parts of native plasma. The prothrombin time was seven seconds on four determinations. Another portion of plasma was added to a tube containing dry ammonium and potassium oxalate in the proportions noted above. The fibrinogen concentration of this plasma was 291 and 287 mg. per 100 ml. on duplicate determinations.

One ml. portions of plasma were placed into pairs of Pyrex test tubes which had been prepared as follows: one pair was treated with silicone and the remaining three pairs were not treated with silicone, but in one pair

150 mg. of crushed glass were added and in another pair 800 mg. The tubes were kept in a waterbath at 37°C and observed for evidences of clotting. After 24 hours the plasma kept in silicone-treated tubes was perfectly clear and showed no evidence of coagulation. Partial clots were observed in the plain glass tubes at the end of 24 hours. The plasma in the tubes containing 150 mg. of crushed glass was solidly clotted in 10 hours, and that in tubes containing 800 mg. of crushed glass was solidly clotted in less than six hours.

At the end of 24 hours the clear plasma in the silicone-treated tubes was transferred to glass tubes containing 800 mg. of crushed glass. No clots were observed during a further incubation period of 24 hours at 37°C. However, the addition of a preparation of rabbit brain thromboplastin caused coagulation to occur in 10 seconds, exactly the same interval required for coagulation of this plasma prior to incubation. On the other hand a portion of this plasma which had been stored at 2°C for 24 hours clotted when exposed to crushed glass at 37°C.

In this experiment virtually platelet-free canine plasma did not clot in silicone-treated tubes after prolonged storage at 37°C, but coagulation did occur in glass tubes. The rate of clotting was directly related to the glass surface area to which the plasma was exposed.

TABLE I

Clotting activity of canine platelet-free plasma

Experiment No.	Whole blood clotting time in glass tubes at 37°C	Native platelet-free plasma				
		Platelets	Clotting time of 1 ml. portions at 37°C			
			Silicone tube	Glass tubes with added crushed glass		
				0	150 mg.	800 mg.
	min.	*per cmm.*	*min.*	*min.*	*min.*	*min.*
1	18	—	∞*	∞	61	12
2	24	3	∞	∞	P.C.	36
3	24	5	∞	∞	13	10
4	22	3	∞	∞	<22 hrs.	11
5	24	1	∞	∞	P.C.	P.C.
6	27	7	∞	∞	12	11
7	28	57	∞	∞	<22 hrs.	20
8	23	68	∞	∞	<22 hrs.	<22 hrs.
9	32	32	∞	∞	P.C.	51
10	17	5	∞	∞	∞	33
11	22	10	∞	∞	12	12
12	23	—	P.C.†	P.C.	14	9
13	20	—	P.C.	P.C.	5	4
14	17	4	P.C.	P.C.	<22 hrs.	<22 hrs.
15	24	5	P.C.	P.C.	7	5
16	36	5	P.C.	P.C.	<22 hrs.	<22 hrs.
17	23	—	<22 hrs.‡	<22 hrs.	<22 hrs.	18
18	25	—	∞	P.C.	14	11
19	24	66	<22 hrs.	P.C.	18	11
20	19	8	∞	P.C.	42	27
21	22	33	∞	40	10	7

* ∞ = No clot at end of 24 hours.
† P.C. = Partial clot at end of 24 hours.
‡ <22 hrs. = Complete clotting occurred at some time between six and 22 hours, but the exact time was not determined.

TABLE II

Clotting activity of canine platelet-free plasma: effect of dilution of plasma with barbital buffer solution

Concentration of plasma	Clotting time of 1 ml. portions at 37°C			
	Silicone tube	Glass tubes with added crushed glass		
		0	150 mg.	800 mg.
per cent	*min.*	*min.*	*min.*	*min.*
100	∞*	∞	<22 hrs.‡	20
80	∞	P.C.†	20	14
50	38	26	14	11
20	36	27	20	14

* ∞ = No clot at end of 24 hours.
† P.C. = Partial clot at end of 24 hours.
‡ <22 hrs. = Complete clotting occurred at some time between six and 22 hours, but the exact time was not determined.

Data of 21 similar experiments are summarized in Table I. In the majority of the experiments there was no difference in the rate and degree of clotting in silicone-treated and in plain glass tubes without added crushed glass. In 11 experiments no clotting occurred in either silicone-treated or plain glass tubes. In six experiments markedly delayed or incomplete clotting, often consisting of only a few fibrin strands, occurred but the rate and degree of clotting were the same in both glass and silicone-treated tubes. These findings are in essential agreement with those of Patton and his associates (9), and in so far as they go might suggest that surface plays no role in the clotting of platelet-free plasma. However, in every instance increasing the glass surface area by the addition of crushed glass caused clotting to occur. The rapidity of clotting was directly related to the amount of crushed glass. In the foregoing experiments there was no relationship between the clotting behavior of the plasma and the precise number of platelets remaining.

When the platelet-free plasma was quickly frozen and then thawed, its clotting activity was not appreciably altered. Such plasma, if not permitted to come in contact with glass at any time, remained fluid when kept for 24 hours at 37°C. However, thawed plasma was readily clotted by the addition of crushed glass. Similarly the clotting properties of platelet-free plasma were retained after storage at 2°C for 24 hours. However, when plasma was stored at 37°C for 24 hours, it could not subsequently be clotted by the

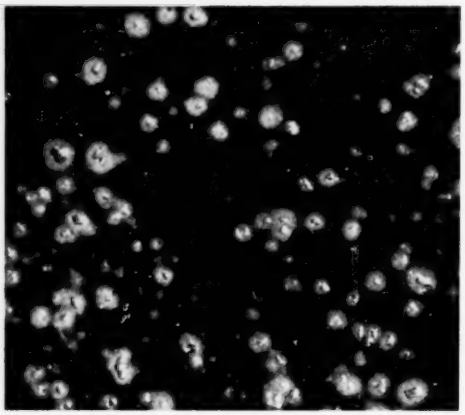

FIG. 1. THE APPEARANCE OF CANINE PLATELET-FREE PLASMA CLOTTING IN CONTACT WITH CRUSHED GLASS

The clots formed about the glass particles, and in the areas between the particles coagulation is not evident. (Enlarged × 6.)

addition of large amounts of crushed glass. Although this stored plasma did not react to the clot-accelerating effect of glass surfaces, it was promptly clotted by the addition of tissue extract thromboplastin.

The effect of dilution of platelet-free plasma with barbital buffer solution was studied. It can be seen from the data shown in Table II that dilution of plasma accelerated the degree and speed of clotting. Plasma which did not clot in silicone-treated and plain glass tubes clotted following dilution with buffer, thus providing additional evidence that canine platelet-free plasma contained all of the components necessary for coagulation. The rate of clotting of diluted plasma was a function of the glass surface area. The barbital buffer solution was prepared in such a manner as to prevent contamination with thromboplastic substances. Furthermore, the addition of this buffer solution to hemophilic platelet-free plasma did not cause clotting to occur.[5] There is thus no evidence that the clot-accelerating effect of dilution with buffer solution was due to inherent thromboplastic properties of the latter.

[5] Hemophilic platelet-free plasma provides a sensitive means for detecting the presence of thromboplastin. Such plasma does not clot spontaneously but is clotted by extraordinarily minute amounts of thromboplastin.

Similar results were obtained when 0.85% sodium chloride solution was used as the diluent.

Direct visual observation of th clotting of canine platelet-free plasma

Clotting of platelet-free plasma was observed using a phase contrast microscope. Glass slides and coverslips were coated with silicone. A few particles of crushed glass were placed on the surface of the glass slide. Platelet-free plasma was introduced between the slide and coverslip and the preparation sealed with vaseline.

In preparations of this type platelets are easily recognized when present. However, in the numerous specimens examined, no platelets were seen. After about 15 minutes a fine fibrin network could be seen at the surface of the glass particles. As clot formation progressed a fibrin collar was formed around each piece of glass. Subsequently this structure became macroscopically visible with a rounded area of clot surrounding each glass particle. In regions remote from the glass the appearance of fibrin was much delayed. Figure 1 shows the appearance of such a preparation.

The clotting activity of platelet-free plasma obtained from a hemophilic dog

Graham and his associates have described the characteristics of a hemorrhagic disease occurring in Irish setters (12). The hereditary pattern, clinical picture, and laboratory data in these animals were identical with those seen in human hemophilia. Opportunity was afforded to study an apparently identical situation in several members of a litter of pure-bred English setters.

The bitch, a vigorous and healthy animal, had had three litters. Some of the males in the first litter were known to have died following the development of multiple hematomas. The second litter consisted of four females and two males, none of which displayed manifestations of a hemorrhagic diathesis. The third litter contained four healthy pups two of which were males. However, three other males showed striking evidence of a hemorrhagic disorder. All developed enormous and apparently painful hematomas, particularly over the back and scalp, and evidence of dysfunction of the hip joints. These manifestations became apparent between the third and sixth weeks of life. Because of their painful disability it was necessary to sacrifice these dogs when they were about three months of age.

TABLE III

Studies on canine hemophilia in two litter mates

	Dog No. 1	Dog No. 2
Whole blood clotting time (min.)	320	>360
Prothrombin time (sec.)	9	9
"Prothrombin consumption"*	Markedly impaired	Markedly impaired
Platelets (per cmm.)	192,000	230,000
Fibrinogen (mg. per 100 ml.)	360	300
Clot retraction	Normal	Normal
Test for circulating anticoagulant (14)	—	Negative

* Modification of the method of Quick and Favre-Gilly (13).

Two of the three animals were available for study, and the results are shown in Table III. These are identical with those seen in human hemophilia, with normal prothrombin, platelet, and fibrinogen concentrations, and negative test for circulating anticoagulant, as well as markedly prolonged whole blood clotting time and impaired "prothrombin consumption."

Platelet-free plasma was prepared from the blood of Dog. No. 2. This plasma failed to clot in silicone-treated or glass tubes. The addition of as much as 800 mg. of crushed glass to 1 ml. of plasma did not cause coagulation to occur. However, the addition of tissue extract thromboplastin brought about rapid clotting. When rabbit brain suspension was added to the platelet-free plasma, coagulation occurred in 10 seconds in duplicate determinations.

DISCUSSION

In these experiments a relatively stable native platelet-free plasma was readily produced from canine blood. In most instances this native plasma failed to clot in silicone-treated tubes even after prolonged incubation at 37°C. Furthermore, canine plasma usually showed no evidence of coagulation when stored in plain glass tubes. However, when the area of glass surface was increased by the addition of crushed glass, clotting invariably occurred, the rate being related to the amount of glass present. When plasma was diluted with buffer solution, coagulation occurred even in silicone-treated tubes. Furthermore, the coagulation of diluted plasma was accelerated by exposure to glass surfaces. These observations demonstrated that those substances necessary for the initiation of coagulation were all present in platelet-free plasma. Careful studies indicated that in the preparation of this plasma no significant changes occurred other than the removal of the formed elements of the blood. Direct microscopic observation provided impressive confirmation of the manner in which glass surfaces initiated coagulation in platelet-free plasma.

In contrast to the behavior of normal canine plasma, hemophilic dog plasma failed to clot on exposure to glass surfaces, although it was as readily clotted by tissue thromboplastin as was normal plasma. This suggests that hemophilic plasma may be deficient in a substance upon which glass surfaces exert their effect.

The observations on dog plasma, while similar to those made on human plasma, showed a significant difference. Canine platelet-free plasma was somewhat more stable than human plasma. It was possible to produce platelet-free plasma which did not clot in silicone-treated tubes more regularly than in experiments on human plasma (7, 8). Furthermore, the amount of glass surface required to bring about coagulation of dog plasma exceeded that necessary for human plasma.

These studies were interpreted as presenting evidence for the existence of a soluble factor in canine as well as human plasma which can *initiate* clotting independent of platelet action. This factor which has been called "plasma thromboplastin" appears to exist in circulating blood in an inactive form which can be activated on contact with glass surfaces (7, 8). In both species the substance which was activated on contact with glass surfaces appeared to be relatively stable at 2°C but lost activity within 24 hours at 37°C.

Platelet-free plasma from hemophilic human subjects clotted not at all or only after great delay on contact with glass surfaces. In many instances the addition of as much as 800 mg. of crushed glass to 1 ml. of platelet-free plasma failed to produce any evidence of clotting (15). In canine hemophilia identical results were obtained. These findings suggest that there is a deficiency of plasma thromboplastin in hemophilia in both species. Plasma thromboplastin may be identical with the antihemophilic globulin of Patek and Stetson (16).

The manner in which glass surfaces activate plasma thromboplastin is unknown. It is possible

that the plasma factor is normally bound to an inhibitor. Such an inhibitor might be adsorbed on glass and other similar surfaces. The acceleration of clotting brought about by the dilution of plasma could be explained by the dilution of an inhibitor. This is a point which has been repeatedly emphasized by Tocantins and others, who have attributed the clotting defect in hemophilia to an excess of such an inhibitor (17–19). We have been unable to demonstrate an excess of an inhibitor in the blood of patients with hemophilia (14, 20). In our experience the dilution of hemophilic platelet-free plasma has not caused coagulation to occur, even in the presence of an excess of crushed glass, in those instances in which the undiluted plasma failed to clot (15, 20). If such an inhibitor does exist, it does not appear to be of the type which can be demonstrated to have an anticoagulant effect on normal blood. However, it is conceivable that an inhibitor is bound to activator and that there is no excess present (18).

While it remains to be proved that a thromboplastin inhibitor is normally present in blood, a specific and easily demonstrable inhibitor of plasma thromboplastin has been found in the blood of certain patients with a hemorrhagic disorder. This substance, which acts as a potent anticoagulant, appears to inhibit only the activation of plasma thromboplastin and has no effect on other stages of blood coagulation. A study of the properties of this anticoagulant has provided further support for the existence of a plasma thromboplastin (21).

The question is often raised whether the effect of glass surfaces on centrifuged plasma might be mediated by the few remaining platelets. In the present experiments the plasmas appeared to be virtually free of platelets. In a previous study on human plasma it was shown that the small number of platelets remaining after centrifugation were probably not concerned with the clot-accelerating effect of glass (8). Furthermore, a globulin fraction prepared from human platelet-free plasma did not clot when protected from contact with glass, but clotted readily when exposed to glass surfaces (22). In this globulin fraction it seems likely that intact platelets were not present.

Although the plasma factor is able to *initiate* coagulation in the absence of platelets, there is no question that the platelets accelerate the conversion of prothrombin to thrombin. For normal clotting both the platelets and plasma factor are required. The relationship between the platelets and plasma factor is not understood. The present studies provide no support for the theory that a platelet enzyme is necessary for the activation of the plasma thromboplastin (23). Neither are these studies compatible with the concept that the plasma factor produces thromboplastin only by exerting a lytic effect on platelets (24). There is no question that platelets, like other cellular structures, contain a small amount of thromboplastin which may eventually be released in shed blood. Thus, hemophilic plasma, presumably deficient in plasma thromboplastin, will not clot in the absence of platelets, but hemophilic plasma containing a normal complement of platelets will eventually clot. It appears that either the plasma thromboplastin or platelets alone can *initiate* coagulation, but for efficient clotting both components are required (7). The reported studies suggest that the clot-accelerating effect of glass surfaces is mediated by the plasma factor rather than by any direct action on platelets. It seems likely that the platelets accelerate the conversion of prothrombin to thrombin after the activation of the plasma thromboplastin has occurred.

SUMMARY

1. Platelet-free plasma was prepared from the blood of dogs without the use of anticoagulants by means of high-speed centrifugation at low temperature using silicone-treated apparatus.

2. Platelet-free plasma obtained from normal dogs usually remained fluid when stored at 37°C in silicone-treated tubes but clotted on contact with crushed glass. The rate of clotting was directly related to the glass surface area.

3. Hemophilic dog plasma from which the platelets had been removed did not clot even when stored in contact with crushed glass.

4. These observations support the view that the clot-accelerating effect of glass surfaces is mediated by a "plasma thromboplastin." This factor is apparently deficient in hemophilia.

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EFFECT OF BENEMID (p-[di-n-PROPYLSULFAMYL]-BENZOIC ACID) ON URATE CLEARANCE AND OTHER DISCRETE RENAL FUNCTIONS IN GOUTY SUBJECTS[1]

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Benemid, (p-[di-n-propylsulfamyl]-benzoic acid), is a potent inhibitor of the renal tubular transport systems responsible for tubular secretion of the penicillins (1–3), p-aminohippurate (1), phenolsulfonphthalein (1) and p-aminosalicylic acid (4). It was initially introduced as an agent more effective than carinamide in sustaining high therapeutic blood levels of penicillin and p-aminosalicylic acid.

Benemid also is a potent uricosuric agent, of low toxicity (5). Gutman and Yü (6), in studies on 39 patients with chronic gout maintained on a low purine, restricted protein diet, found that Benemid consistently caused a rapid and marked increase in urinary urate excretion and a sharp decline in serum urate levels. The mean rise in urinary urate excretion in the first week of Benemid administration was 67%, 52% and 46% with daily doses of 2 gm., 1 gm. or 0.5 gm., respectively, resulting in corresponding falls in serum urate of 42%, 33% and 27%. Talbott, Bishop, Norcross, and Lockie (7) have reported similar results. The drug appears to have an important place in the management of chronic gout (8).

In the present study the renal mechanisms whereby the uricosuric action of Benemid is effected have been investigated. Simultaneous renal clearance studies of urate, inulin or endogenous creatinine, p-aminohippurate, sodium, potassium and chloride were carried out in 10 male gouty subjects before and after oral administration of 2 gm. of Benemid. In some of these subjects the effects of Benemid on phosphate clearance, urinary excretion of hydrogen and ammonium ion, and on urine and blood pH were also studied. The implications of our findings in respect to the renal mechanisms for regulation of urate excretion by the kidneys in normal and gouty man are discussed.

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METHODS

The subjects, whose ages ranged from 38 to 54 years, were all known to have had gout for several years. Only patients without overt cardiovascular and renal disease (no albuminuria, cylindruria, nitrogen retention) were used for these experiments. All had been on low purine, restricted protein (50–80 gm./day) diets preceding study and were in the morning post-absorptive state at the time of the experiment. Water was ingested in liberal quantities to maintain urine flow rates above 4 ml./min. throughout the experiment.

After drawing blood for inulin and p-aminohippurate plasma blank values, priming and sustaining infusions of these substances in 0.89% saline were administered by means of a Bowman constant infusion pump at rates calculated to maintain plasma concentrations of inulin at 30 mg.% and p-aminohippurate at 2 mg.%. Thirty minutes were allowed for equilibration. Bladder catheterization was then performed with multi-eyed hard rubber catheters and three 10- to 15-minute urine collections were obtained for the control clearance values. Bloods were drawn midway between the first and third clearance periods. Following the third control period 2 gm. of the drug were administered orally (19.3 to 29.5 mg./kg.). Six 20-minute post-Benemid clearance periods were then obtained. Three bloods were drawn approximately 30, 70 and 110 minutes after drug administration and representative plasma concentrations of inulin, urate and p-aminohippurate were obtained by plotting the concentrations semilogarithmically against time and by extrapolating back to 2.5 minutes (approximate urine delay time) from the mid-time of each urine collection period. Bladder washouts with 20 ml. of distilled water, followed by air, effected complete emptying at the termination of each urine collection period. With slight modifications the clearance techniques employed were those recommended by Goldring and Chasis (9).

In two additional subjects simultaneous endogenous creatinine, urate, sodium, potassium and chloride clearances were obtained at 30-minute periods for two hours preceding and two hours following administration of Benemid. In this manner intravenous infusions were

avoided and urine could be collected by spontaneous voiding.

In two gouty subjects the effects of a single 2 gm. oral dose of Benemid upon simultaneous inulin, urate, endogenous creatinine and p-aminohippurate were studied over 24 hours. The drug was administered to these subjects following three 10- to 20-minute control periods, during which time urine was collected by catheter and bladder wash. Following administration of the drug, urines were collected by spontaneous voiding at two-hour intervals during the day and at two four-hour intervals at night. Intravenous infusions of inulin and p-aminohippurate were maintained for 24 hours employing the technique of Sirota, Baldwin and Villarreal (10). Two bloods were drawn during the control periods, and six at strategically spaced intervals for 24 hours after drug ingestion. In a third subject the diurnal variations of endogenous creatinine, urate, sodium, potassium and chloride clearances were studied for 48 hours preceding Benemid and for 48 hours following a single 2 gm. oral dose. This patient was hospitalized and maintained on a constant protein (50 gm.), constant salt (3.5 gm.) diet for three days preceding the control study. Water was allowed *ad lib.* throughout.

TABLE I

The acute effect of oral Benemid upon the renal clearances of inulin, urate and PAH in 10 subjects with non-tophaceous gout

Subject Age	Period	Renal clearances‡					Plasma uric acid
		C_{IN}	C_{URATE}	C_{PAH}	C_{URATE}/C_{IN}	C_{IN}/C_{PAH}	
		ml./min.	*ml./min.*	*ml./min.*			*mg. %*
	Control*	100	5.02	557	0.050	0.179	6.7
	Benemid 24.6 mg./kg.						
	20 min.	103	5.39	618	0.052	0.167	
A. R.	40 min.	108	14.5	590	0.135	0.183	6.8
41	60 min.	109	24.3	632	0.223	0.173	
	80 min.	109	25.8	455	0.237	0.239	6.6
	100 min.	116	29.9	395	0.258	0.294	
	120 min.	107	27.3	520	0.255	0.205	6.2
	Control	117	8.30	529	0.071	0.221	11.0
	Benemid 19.3 mg./kg.						
	20 min.	131	9.00	508	0.069	0.242	
H. G.	40 min.	112	8.00	576	0.071	0.195	11.1
38	60 min.	119	18.1	638	0.152	0.187	
	80 min.	119	33.6	634	0.282	0.188	10.3
	100 min.	113	27.2	341	0.241	0.332	
	120 min.	104	13.2	311	0.127	0.335	10.3
	Control	119	11.0	610	0.092	0.195	8.2
	Benemid 25.0 mg./kg.						
	20 min.	119	11.8	579	0.099	0.220	
L. G.	40 min.	122	28.8	538	0.236	0.230	8.1
54	60 min.	121	36.7	516	0.303	0.209	
	80 min.	115	36.6	463	0.318	0.268	7.5
	100 min.	122	40.2	480	0.330	0.214	
	120 min.	113	42.1	414	0.373	0.261	6.8
	Control	99.9	9.14	621	0.092	0.161	7.3
	Benemid 27.5 mg./kg.						
	20 min.	92.0	10.1	640	0.110	0.144	
H. H.†	40 min.	94.0	16.3	582	0.174	0.162	7.2
47	60 min.	77.7	13.6	520	0.175	0.149	
	80 min.	85.4	31.3	522	0.367	0.164	6.4
	100 min.	67.8	27.4	388	0.404	0.175	
	120 min.	82.4	35.3	502	0.428	0.164	6.2
	Control	137	13.2	623	0.096	0.220	8.0
	Benemid 23.2 mg./kg.						
	20 min.	132	13.8	656	0.105	0.201	
W. L.†	40 min.	136	21.2	632	0.156	0.215	7.8
42	60 min.	121	32.0	510	0.265	0.237	
	80 min.	130	51.0	504	0.393	0.258	7.2
	100 min.	129	51.3	377	0.398	0.343	
	120 min.	110	51.2	299	0.465	0.369	6.5

* The control clearance values represent the averages of three 10- to 15-minute urine collection periods. The control plasma urate values represent the averages of three separate determinations before administration of Benemid.

† Endogenous creatinine clearances were substituted for inulin clearances in these instances.

‡ Not corrected for surface area (values in text so corrected are indicated there).

TABLE I—*Continued*

Subject Age	Period	Renal clearances					Plasma uric acid
		C_{IN}	C_{URATE}	C_{PAH}	C_{URATE}/C_{IN}	C_{IN}/C_{PAH}	
		ml./min.	*ml./min.*	*ml./min.*			*mg. %*
	Control	83.7	7.68	332	0.092	0.253	9.6
	Benemid 25.5 mg./kg.						
	20 min.	90.7	12.2	343	0.135	0.264	
	40 min.	92.6	45.7	328	0.493	0.282	8.7
N. W.	60 min.	88.8	48.0	244	0.541	0.364	
54	80 min.	72.0	39.4	203	0.547	0.355	8.5
	100 min.	103	50.9	125	0.493	0.825	
	120 min.	69.4	28.7	355	0.413	0.195	7.6
	Control	76.0	6.62	485	0.087	0.157	6.3
	Benemid 29.5 mg./kg.						
	20 min.	76.6	7.50	448	0.098	0.171	
M. M.	40 min.	81.0	11.5	457	0.142	0.177	6.5
53	60 min.	84.0	14.3	434	0.170	0.194	
	80 min.	68.5	13.7	380	0.200	0.180	6.1
	100 min.	72.4	14.1	383	0.195	0.189	
	120 min.	79.3	18.1	426	0.228	0.186	5.8
	Control	128	13.2	650	0.103	0.197	9.5
	Benemid 23.3 mg./kg.						
	20 min.	138	14.0	664	0.100	0.208	
A. L.	40 min.	124	23.0	584	0.184	0.213	9.6
45	60 min.	123	33.6	446	0.270	0.363	
	80 min.	113	31.1	566	0.272	0.200	
	100 min.	108	31.4	518	0.307	0.208	
	120 min.	116	29.6	524	0.252	0.221	8.0
	Control	98.5	4.98	526	0.051	0.187	8.0
	Benemid 22.7 mg./kg.						
	20 min.	110	4.60	516	0.042	0.213	
J. R.	40 min.	95.8	3.79	489	0.040	0.196	8.3
39	60 min.	90.1	4.34	443	0.048	0.203	
	80 min.	95.6	4.84	462	0.051	0.207	8.1
	100 min.	91.2	6.29	469	0.069	0.197	
	120 min.	87.9	10.4	432	0.118	0.203	8.0
	Control	75.1	6.32	316	0.084	0.238	13.9
	Benemid 26.2 mg./kg.						
	20 min.	78.2	7.80	342	0.100	0.229	
R. C.	40 min.	76.2	12.1	348	0.159	0.219	12.6
47	60 min.	71.2	23.6	268	0.332	0.266	
	80 min.	73.0	16.6	251	0.228	0.291	12.4
	100 min.	70.1	17.1	253	0.244	0.277	
	120 min.	67.7	20.6	238	0.304	0.284	11.2

Inulin in plasma and urine was determined by the method of Schreiner (11), p-aminohippurate by the method of Smith and his associates (12), "creatinine" by the method of Bonsnes and Taussky (13), phosphate by the method of Fiske and SubbaRow (14) as modified for spectrophotometry, and chloride by either the method of Schales and Schales (15) or Van Slyke and Hiller's modification of the Sendroy method (16). Sodium and potassium analyses were performed by means of the Perkin-Elmer internal standard flame photometer, Model 120 A. Urine ammonia determinations were performed by the method of Van Slyke and Cullen (17), urine titratable acidity by the technique of Pitts and Alexander (18), and blood and urine pH by means of the Cambridge pH meter. Plasma and urine urate concentrations were determined by a modification of the method of Buchanan, Block and Christman (19), using uricase digestion, urea cyanide-carbonate and arsenophosphotungstic acid. With this method inulin and p-aminohippurate, in the concentration ranges present in these studies, were found not to interfere with the recovery of urate from plasma and urine. For estimating filtrable plasma urate, ultrafiltrates of plasma were obtained by means of the Simms-Sanders "surge" ultrafiltration apparatus (20). In this device negative pressures are applied and rapidly varied by 10–15 mm. Hg so as to remove the serum protein film which would otherwise soon occlude the surface of the collodion sac. Thus the rate of ultrafiltration is greatly increased and a minimum of urate is retained by adsorption on the protein film.

RESULTS

Acute experiments. Table I summarizes observations on the acute effects of 2 gm. Benemid given orally on the renal clearances of inulin (C_{IN}), urate (C_{URATE}) and p-aminohippurate (C_{PAH}) in 10 gouty subjects. Despite the absence of gross manifestations of renal damage in these patients, the control C_{IN} and C_{PAH} values were found to be slightly to moderately depressed. Thus the mean C_{IN} was 92.5 ml./min./1.73 M^2 with a range of 73–111; the mean C_{PAH} was 472 ml./min./1.73 M^2 with a range of 307–596.

As indicated in Table I, Benemid in this dosage consistently caused a striking increase in C_{URATE} which was sustained over the two-hour period of observation. C_{URATE} rose from a mean control value of 8.55 ml./min. (range 4.98–13.2) to peak values averaging 32.9 ml./min. (range 10.2–51.3). In eight of the 10 subjects this rise was apparent within 40 minutes of ingestion of the drug. In six instances an accompanying fall in plasma urate of 1 mg.% or more was noted by the end of two hours.

Significant changes in C_{IN} did not occur. Consequently, the ratio C_{URATE}/C_{IN} rose, from a mean control value of 0.071 (range 0.050 to 0.103) to peak values averaging 0.337 (range 0.118 to 0.547). In two cases (not included in Table I) in which the clearance of endogenous creatinine was used as a measure of glomerular filtration and intravenous infusions thus avoided, C_{URATE}/C_{CR} rose from control values of 0.066 and 0.046 to 0.510 and 0.252, respectively, after Benemid. The C_{CR}/C_{IN} ratio was found to average 1.03 ± 0.03 before Benemid and 0.965 ± 0.048 after Benemid in eight gouty subjects, a correspondence which justifies use of C_{CR} for GFR under these circumstances.

TABLE II

The effect of oral Benemid upon the renal clearances of sodium, potassium, chloride and phosphate in gouty subjects

Case	C_{IN}			$100 \times \frac{C_{Na}}{C_{IN}}$			$100 \times \frac{C_{Cl}}{C_{IN}}$			$100 \times \frac{C_{K}}{C_{IN}}$			$100 \times \frac{C_{PO_4}}{C_{IN}}$		
	Control	After Benemid	% Change	Control	After Benemid	% Change	Control	After Benemid	% Change	Control	After Benemid	% Change	Control	After Benemid	% Change
R. C.	75.1	72.7	−3.3	4.26	4.15	−2.6	5.51	5.19	−5.8	20.0	18.7	−6.5	9.64	9.29	−3.6
L. G.	119	119	0.0	2.98	3.66	+22.7†	3.35	4.14	+23.6†	11.1	17.3	+55.8	19.0	22.6	+19.0
J. R.	98.5	95.1	−3.6	1.46	2.01	+37.7	1.95	2.57	+31.8	29.0	25.2	−13.1	21.0	22.1	+52
A. L.	128	120	−6.2	2.67	3.09	+15.9†	2.19	2.89	+32.0	22.0	17.6	−20.0	17.0	14.9	−12.3
H. H.*	99.9	83.2	−16.8	6.33	5.87	−7.3	8.03	5.70	−29.0	38.4	24.1	−37.2	8.34	6.36	−23.8
W. L.*	137	126	−8.0	1.98	2.84	+43.5†	1.56	2.18	+39.6†	25.8	24.2	−6.2	10.9	10.9	0.0
H. G.	117	113	−3.4	1.71	1.85	+8.2	2.57	2.62	+2.0	34.7	31.0	−10.7			
M. M.	76.0	77.1	+1.5	2.30	2.79	+21.3	3.05	3.38	+10.8	25.2	26.6	+5.6			
A. R.	100	108	+8.0	1.05	2.41	+130†	1.64	3.24	+97.2†	47.4	29.2	−38.4			
N. W.	83.7	86.1	+3.0	3.26	6.76	+107†	3.46	5.15	+48.8	20.5	23.0	+12.2			
O. G.*	83.5	70.6	−15.4	0.27	0.59	+119	0.78	1.50	+92.5†	24.8	32.0	+29.0			
A. R.*	120	111	−7.5	2.10	1.70	−19.0	1.38	1.12	−18.8	10.9	14.2	+30.3			
Mean S.D.			−4.3 ±2.1			+39.7 ±14.7			+24.1 ±11.4			+.07 ±8.1			−2.6 ±5.9

The control values represent the averages of three 10- to 15-minute periods. The values after Benemid represent the averages of six 20-minute periods starting immediately after oral ingestion of 2 gm. of the drug.

* C_{CR}, endogenous creatinine clearance, used instead of C_{IN} in these subjects.

† These changes considered significant ($p < .02$). The t test of significance for the $\frac{C_{Na}}{C_{IN}}$ and $\frac{C_{Cl}}{C_{IN}}$ ratios was applied to the six post-Benemid clearances as compared to the three control periods for each of the subjects.

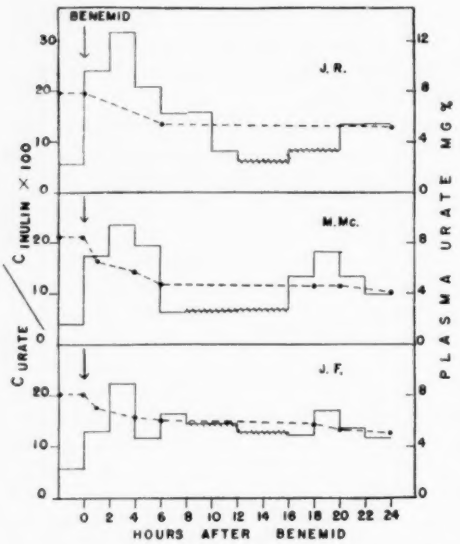

FIG. 1. C_{URATE}/C_{IN} RATIOS AND PLASMA URATE CONCENTRATIONS IN THREE SUBJECTS FOR 24 HOURS FOLLOWING A SINGLE ORAL DOSE OF 2 GM. BENEMID (ARROW)

C_{CR} was substituted for C_{IN} in subject J. R. The pre-Benemid control C_{URATE}/C_{IN} ratios represent the average of three 10- to 15-minute periods. The solid lines represent the C_{URATE}/C_{IN} ratios, the interrupted lines plasma urate concentrations and cross-hatches the sleeping hours.

There was a general moderate decline in C_{PAH} usually evident after one hour. The clearance ratios C_{IN}/C_{PAH} rose from a mean control value of 0.201 (range 0.157 to 0.253) to peak values averaging 0.333 (range 0.175 to 0.825).

Table II summarizes observations on the clearances of sodium (C_{Na}), potassium (C_K), chloride (C_{Cl}) and phosphate (C_{PO_4}) in these gouty subjects. Benemid was found to have negligible effects on C_K and C_{PO_4} and on the ratios C_K/C_{IN} and C_{PO_4}/C_{IN}; the mean per cent changes during the two-hour period following drug administration for C_{IN} were $-4.3 \pm 2.1\%$; for C_K/C_{IN}, $+0.7 \pm 8.1\%$; for C_{PO_4}/C_{IN}, $-2.6 \pm 5.9\%$. The mean per cent changes for C_{Na}/C_{IN} and C_{Cl}/C_{IN} were of much greater magnitude, $+39.7 \pm 14.7\%$ and $24.1 \pm 11.4\%$, respectively. Statistical analysis indicates significant increases in both C_{Na}/C_{IN} and C_{Cl}/C_{IN} ratios in four of the 12 subjects, a significant rise in C_{Na}/C_{IN} only in a fifth subject, and in C_{Cl}/C_{IN} only in a sixth subject after Benemid.

In the two patients so studied there were no significant effects of the drug upon urine or blood pH, urine titratable acidity and ammonium ion excretion. However, these latter observations were obtained without previously rendering the subjects acidotic, and with low control values (2 to 10 μeq. hydrogen ion/min. and 10 to 19 μeq. ammonium ion/min.).

Prolonged experiments. Since the two-hour experiments lapsed before the uricosuric effect of Benemid had worn off, observations were prolonged over a 24-hour period in three gouty subjects in an attempt to establish the duration of increased C_{URATE} following ingestion of a single 2 gm. dose (Figure 1). The peak effect upon C_{URATE}/C_{IN} or C_{URATE}/C_{CR} ratios was found within the first four hours; in Case J. R. rising from a mean control ratio of 0.056 to 0.320, in Case M. Mc. from 0.034 to 0.235, and in Case J. F. from 0.058 to 0.225. Significant elevations of these ratios persisted for 24 hours with a diurnal depression during sleeping hours. At the end of 24 hours the plasma urate concentrations had fallen from 7.9 to 5.1, 8.4 to 4.2, and 8.2 to 4.1 mg. %, respectively.

The results of a four-day study of the excretion of urate, water, sodium, potassium, chloride

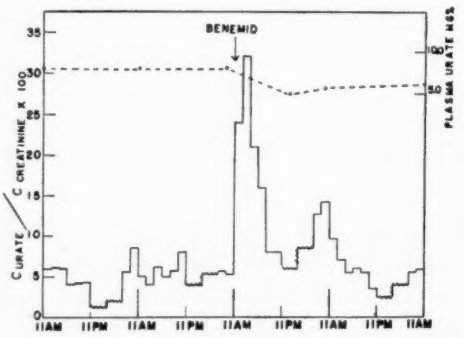

FIG. 2. C_{URATE}/C_{CR} RATIOS AND PLASMA URATE CONCENTRATIONS IN ONE SUBJECT FOR 48 HOURS PRECEDING AND FOLLOWING A SINGLE 2 GM. DOSE OF BENEMID WHILE ON A CONSTANT DIET

The solid line represents the C_{URATE}/C_{CR} ratios; the interrupted line plasma urate concentrations, and the cross-hatches the sleeping hours.

and creatinine in one gouty subject before and after a single dose of Benemid while on a controlled protein (50 gm.), constant salt (3.5 gm.), water *ad lib.* regimen are presented in Figure 2. The subject was maintained on this constant diet for three days before obtaining the two-day pre-Benemid control data. The C_{URATE}/C_{CR} ratios during the two control days and the second post-medication day showed diurnal depressions during sleeping hours similar to those while under Benemid influence. The 24-hour urate excretion rose to 1143 mg. from control values of 543 and 502 mg. This was associated with depression in the plasma urate concentration from a premedication value of 7.9 mg. % to 5.1 mg. %, the greatest decrement occurring during the first four hours. There were no significant changes in the 24-hour excretion of sodium, potassium, chloride, water and creatinine following drug administration.

DISCUSSION

The current concept of renal mechanisms for urate excretion in normal man, recently summarized by Homer Smith (21), assumes complete filtration at the glomerulus and subsequent tubular reabsorption of all but a small fraction of the filtered urate, of which only 5–10% appears in the urine. The tubular transport mechanisms for reabsorption of urate have been shown to be of limited capacity (22) with a Tm_{URATE} of approximately 15 mg./min., and, while not identified, are presumed to be enzymatic. There is no evidence for tubular secretion of urate in normal man, although this is known to occur in birds and reptiles.

In connection with the question of tubular secretion of urate in man, Praetorius and Kirk (23) report the case of a young man, in apparent good health, who was found to have a very low plasma urate (0.2 to 0.6 mg.%) associated with elevated C_{URATE}/C_{IN} ratios averaging 1.46. The authors interpreted these high ratios as indicating tubular secretion of urate. Although the renal clearance of urate undoubtedly was very high in this individual, the C_{URATE}/C_{IN} ratios must be interpreted cautiously because of the analytical errors inherent in available methods for estimating such low plasma concentrations of urate and of inulin (9.03 to 9.77 mg.%) as were present during their study. The plasma blank for inulin averages 1 to 2 mg./ml., 10–20% of the total plasma value.

We have had opportunity to study an unusual case of marked glomerulo-tubular imbalance of the Fanconi type, to be reported in detail elsewhere, which appears to throw light on this problem. In this subject C_{IN} was markedly depressed (26.1 ml./min.) but the functions attributed to proximal tubular activity were disproportionately and quite extraordinarily low: C_{PAH} 36.7 ml./min., Tm_{PAH} 4.9 mg./min., Tm_{G} 22.0 mg./min. The plasma urate (2.3 to 2.8 mg.%) was consistently below the normal range for the method we employ (4–6 mg.% in males) yet the urate clearance was consistently elevated (C_{URATE} 25.7 ml./min. as compared with the normal of about 8 ml./min.) and the urinary urate excretion was about twice normal (0.75 mg./min. instead of 0.30 to 0.40 mg./min.).

In view of the indications of severe tubular damage in this patient, and the evidences of impaired reabsorption of glucose (renal glycosuria), amino acids (amino aciduria) and phosphate (hyperphosphaturia), it seemed more rational to ascribe the high urinary urate excretion to impaired tubular reabsorption of urate than to enhanced tubular secretion of urate. This interpretation was supported by the finding in this patient of a C_{URATE}/C_{IN} ratio of approximate unity (0.984), as compared with a normal mean C_{URATE}/C_{IN} ratio of 0.067, which would indicate both complete filtrability at the glomerulus and virtual absence of urate reabsorption by the tubules. In view of the concurrent evidence in this patient of generally reduced function of the proximal tubules, it may be inferred that urate normally is reabsorbed wholly or in large part by the proximal convoluted tubules. The fact that C_{URATE}/C_{IN} did not exceed unity argues against tubular secretion of urate.

In accord with these inferences is the striking fact that of all the non-gouty and gouty subjects to whom we have administered Benemid, this patient was the only one in whom the drug failed to evoke increased urinary urate excretion and increased urate clearance.

The renal mechanisms for urate excretion in gouty subjects do not appear to differ in any essential from those of normal man (24, 25). However, in view of the often marked hyperuricemia in gout, complete filtrability of urate at the glomerulus cannot be assumed. We therefore subjected the sera of four of these patients, before treatment with Benemid, to ultrafiltration. The results, in conformity with those obtained in 13 other cases of gout (26), showed the serum urate to be wholly filtrable, apart from variations within the limits of error of the method: in case A. R., serum urate 11.3 mg. % (expressed in terms of serum water), ultrafiltrate urate 11.6 mg. %; in Case O. G., serum urate 11.1 mg. %, ultrafiltrate 10.6 mg. %; in Case M. M., serum urate 8.7 mg. %, ultrafiltrate 8.8 mg. %; in Case H. H., serum urate 8.5 mg. %, ultrafiltrate 7.8 mg. %. In view of these findings tubular reabsorption of

urate and not non-filtrability of plasma urate must be assumed to explain the large discrepancy between urate clearance and glomerular filtration rate in these patients. The possibility of some tubular excretion of urate in gout is not excluded.

Saturation of tubule reabsorptive capacity for urate probably does not occur either in normal subjects (22) or in gouty patients with adequate renal function. According to Berliner, Hilton, Yü and Kennedy (22), a urate load/Tm_{URATE} ratio of 1.25–1.50 is necessary for saturation which, with a normal Tm_{URATE} of 15 mg./min., would require filtered urate loads between 18.9 and 22.5 mg./min.—at a normal filtration rate of 120 ml./min., necessitating plasma urate concentrations of 15.8–18.7 mg. %. Since the filtration rate was depressed in most of our gouty patients, even higher plasma urate concentrations would be required. The greatest filtered urate load recorded in our gouty subjects was 14.5 mg./min. (Patient H. G., Table I). Moreover, no increase in C_{URATE}/C_{IN} ratios was observed in the patients with greater filtered urate loads, such as might be expected if significant tubule saturation had occurred.

There is every indication that, as in its effects on penicillin and p-aminosalicylic acid, Benemid produces uricosuria primarily by altering renal excretory mechanisms. The rapidity of the uricosuric effect, within 40 minutes of ingestion in most instances, makes it highly improbable that it can be accounted for by increased breakdown of nucleic acids, which normally have a slow turnover rate, or by accelerated urate biosynthesis. Moreover, studies carried out in two of our gouty subjects before, during and after ingestion of 2 gm. of Benemid showed no concurrent increase in urinary total nitrogen or phosphorus excretion (26). Bishop, Rand and Talbott (27) have demonstrated a decrease in the miscible pool of urate after administration of Benemid, indicating a predominantly excretory effect of the drug.

Our data indicate clearly that the enhancement of renal excretion of urate by Benemid is not due to an increase in glomerular filtration but is attributable to inhibition of tubular reabsorption of urate. In none of the subjects studied by us was this inhibition complete, the highest C_{URATE}/C_{IN} ratio obtained being 0.547. Since the drug exerts its effect upon known proximal tubule function (PSP, PAH, PAS and penicillin secretion) it appears likely that the uricosuric effect is mediated at this level of the nephron. It is interesting that the uricosuria produced by Benemid is the result of inhibition of a reabsorptive process whereas its other known effects are based upon inhibition of secretory mechanisms. The drug appeared to exert no effect upon phosphate reabsorption, the only other reabsorptive process with a Tm mechanism studied by us. Beyer and his associates (1), working with dogs, demonstrated that the drug exerted no effect upon the reabsorptive mechanisms for glucose, arginine, sulfonamides, urea and phosphate. The precise mechanism of the inhibitory effect of Benemid on tubular urate reabsorption has not been determined. According to Beyer and co-workers (1), the drug, in concentrations which suppress PAH, PSP, PAS, penicillin and urate transport, will inhibit utilization of energy from high-energy phosphate bonds without interfering with oxygen uptake or phosphorylation.

Significant depression of C_{PAH} occurred following Benemid admininstration in eight of the 10 subjects so studied. From the data in Table I it is impossible to indicate to what extent these depressions resulted solely from inhibition of tubule secretory activity, since decrease in renal plasma flow might partially account for C_{PAH} depressions. Quantitative evaluation of the effect of the drug upon this tubule function is possible only by PAH renal extraction and Tm_{PAH} measurements. Beyer and his associates (1), employing Benemid in approximately twice the relative dosage used in our studies, were able to reduce Tm_{PAH} to almost zero in dogs. In one non-gouty subject without renal disease studied by us, Benemid in a dose of 20 mg./kg. produced a fall of Tm_{PAH} from 83.4 mg./min. to 26.4 mg./min. without influencing C_{IN}, suggesting that the depressions in C_{PAH} observed were primarily the result of inhibition of tubule secretory activity.

In the acute studies several of the subjects showed significant increases in the clearances of either sodium or chloride, or in both of these ions. However, the absolute magnitude of the renal clearances of these ions was so low that a slight increment would result in a marked increase in

their clearance when expressed as per cent change. Thus, the maximum per cent change in C_{Na}/C_{IN} noted, 130% (Case A. R., Table II), represents an actual increment of C_{Na} from 1.05 to 2.60 ml./min., and a change in urine excretion of 0.147 to 0.364 meq./min. The subjects who exhibited this increase in C_{Na} and C_{Cl} were among those who presented the most marked uricosuria. Significant water diuresis did not occur in these studies. However, the control urine flows before administration of the drug were maintained at high levels and may have masked a diuretic effect. The one subject studied on the controlled purine and salt intake and water *ad lib.* failed to exhibit an increase in either salt or water output for the 24-hour period following drug ingestion.

Beyer and co-workers (1) found that less than 2% of Benemid is excreted in dog's urine during 24 hours after a single intravenous dose, and that determinable plasma concentrations of the drug were present 48 hours after a single dose of 60 mg./kg. They found that approximately 75% of the drug was bound to plasma protein and 25% was freely filtrable. This latter fraction is presumably filtered at the glomerulus and is virtually completely reabsorbed by the tubules. This provides an explanation for the prolonged uricosuric effect following a single dose of the drug. An oral dose of approximately 20 mg./kg. produced elevations of C_{URATE}/GFR above control levels for 24 hours in the three subjects so studied. The complete tubular reabsorption of filtered Benemid presumably results in the maintenance of an effective concentration in or about the tubule cells for a prolonged period of time.

The diurnal variation of renal urate clearance noted in our cases is of some interest. Brøchner-Mortensen (28) reported a diurnal variation in urate clearances, with the lowest values occurring at night. Leathes (29) studied the diurnal urate excretion in a single individual on two different levels of purine ingestion and found decreased excretion during the sleeping hours on both levels of purine intake. Stanbury and Thomson (30) demonstrated a relatively greater depression of urate excretion than GFR in a single individual during sleeping hours. C_{URATE} was not determined in this subject. Since our studies demonstrated a fall in C_{URATE}/GFR ratios at night, the decreased clearance of urate cannot be explained solely by the decreased glomerular activity which usually occurs at night (10). Benemid does not alter this diurnal pattern, even though the nocturnal C_{URATE}/GFR ratios, while under drug influence, are still higher than the control values. This pattern apparently reflects diurnal variation in tubule activity with respect to urate reabsorption. It is well established that a similar pattern occurs for the renal excretion of the major electrolytes and water (30).

Unless water diuresis is encouraged during Benemid uricosuria excessively high concentrations of urate may be encountered in the urine. Thus, in one of our subjects on the 24-hour study maintained on fluids *ad lib.*, a concentration of urine urate of 157 mg. % was obtained at a time when his urine flow was less than 1 ml./min. Talbott and Coombs (31) found a maximum urate solubility of slightly more than 100 mg./100 ml. in the urine of gouty subjects. Alkalinization and water diuresis would therefore appear to be important measures for the prevention of uric acid crystalluria during Benemid therapy. In a gouty subject with normal glomerular function and a plasma urate concentration of 8 mg. %, a urine flow of at least 4 ml./min. is necessary to maintain urine urate concentrations under 100 mg./100 ml. during the peak of Benemid uricosuria, which occurs within four hours after drug administration.

No indication of toxicity attributable to Benemid was observed in the course of these experiments, with the possible exception of one patient who developed an acute attack of gout the day after. The effects upon renal transport mechanisms are completely reversible following discontinuance of medication. Benemid appears thus far to be of a low order of toxicity (6, 32) and no overt signs of renal "irritation" (albuminuria, casts, red blood cells) unequivocally attributable to the drug have yet been reported.

SUMMARY

1. In 15 clearance studies carried out in 13 gouty subjects, Benemid given orally in a single 2 gm. dose consistently produced a marked rise in urate clearance, from a mean control level of 7.99 ml./min. to a mean peak value of 32.7 ml./

min. This rise was usually evident within 40 minutes of drug administration and lasted 24 hours in the subjects followed for this period. The plasma urate level fell concomitantly.

2. There was no significant effect on glomerular filtration rate. The ratio C_{URATE}/GFR consequently rose, from a mean control value of 0.0718 to peak values averaging 0.333.

3. Some depression of C_{PAH}, with consequent moderate rise in the ratio C_{IN}/P_{PAH}, was observed in most instances.

4. Phosphate and potassium clearances were unaffected by Benemid. Slight but significant increases in sodium or chloride clearance, or both, occurred in about half the subjects.

5. A marked diurnal variation in C_{URATE}/GFR ratios was noted in those subjects in whom these functions were studied over one or more 24-hour periods. The depression of these ratios noted during sleeping hours persisted in spite of continued overall Benemid uricosuria.

6. The data obtained support the complete filtration-partial tubular reabsorption concept of urate excretion in the normal and gouty subject and indicate that Benemid uricosuria is the result of a highly selective inhibition of tubular reabsorption of filtered urate. Corroboratory evidence for this conclusion was derived by clearance studies in one subject with glomerulo-tubular imbalance of the Fanconi type who was found to have a C_{URATE}/C_{IN} ratio of approximate unity, which was uninfluenced by Benemid.

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EXPERIMENTAL ASCITES. STUDIES OF ELECTROLYTE BALANCE IN DOGS WITH PARTIAL AND COMPLETE OCCLUSION OF THE PORTAL VEIN AND OF THE VENA CAVA ABOVE AND BELOW THE LIVER

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Previous studies (1–6) of experimental ascites primarily concerned with protein metabolism and exchange have emphasized the importance of sodium in ascitic fluid production. Recently, the importance of sodium retention in several forms of effusions and edema has been described by numerous observers (7–15) who have all directly or indirectly implicated the kidney in the causal relationships of this abnormality of sodium metabolism. This report concerns sodium, potassium, and chloride balance studies in dogs with experimentally produced ascites. Following the administration of large amounts of sodium, these ascitic animals, in contrast to the normal dog, do not excrete the excess sodium in their urine. Whereas normally in the dog there is a slightly delayed excretion of sodium with temporary retention of sodium in extracellular and intracellular spaces, these ascitic animals produce predictable amounts of ascites following an increased salt load. Potassium is promptly excreted by the kidneys and chloride is retained only when there is sodium retention. The ascites of these animals seems to resemble in some ways that of the human cirrhotic and the patient with right sided heart failure or chronic constrictive pericarditis. The *liver* seems *implicated* in this abnormality of *sodium metabolism.*

EXPERIMENTAL METHODS

Healthy mongrel dogs averaging 8 to 12 kilograms in weight were selected for these experiments following observation for several weeks under standard conditions in the animal house. Aluminum bands were used for partial and complete occlusion of the portal vein and the vena cava below and above the liver by techniques previously described (2). Two animals were studied before and after partial and complete occlusion of the portal vein and the vena cava below the liver and above the kidneys. A third animal was studied before and after three operative procedures that first partially, then completely, occluded the portal vein and the vena cava below the liver and above the kidneys, and finally partially occluded the vena cava above the liver. The fourth and fifth animals were studied following partial occlusion of the inferior vena cava above the liver.

During these periods of observation the animals were maintained in metabolism cages with a constant daily diet of horsemeat, 200 or 250 grams, and 50 grams of a low protein mixture (16) that contained sodium, potassium, chloride, and nitrogen in amounts indicated (Table I). The daily dietary intake, therefore, was 14.5 milliequivalents of potassium, 8.5 milliequivalents of sodium, and 5.6 milliequivalents of chloride for animal 50–5, and was 17.0 milliequivalents of potassium, 9.0 milliequivalents of sodium, and 6.4 milliequivalents of chloride for the other experimental animals. Additional sodium chloride and potassium (neocurtasal) [1] were given orally in capsules in large amounts at appropriate periods indicated in the accompanying graphs. Water was allowed *ad libitum.* During these periods complete balance studies of sodium, potassium, chloride, water, and protein were carried out. The animals were carefully weighed at the same time each day. Determinations of plasma protein and ascitic fluid protein were made by methods previously described (1). A Perkin-Elmer flame photometer, model 52-A, supplied with an acetylene burner was used to determine potassium and sodium utilizing lithium as an internal standard by the method previously described (17). No significant interference was noted from neighboring bands of other elements. Chlorides were determined by the method of Van Slyke (18).

EXPERIMENTAL RESULTS

The accompanying figures summarize the balance studies carried out on our experimental animals. Nitrogen balances, though carried out, were omitted from the graphs as similar studies have been reported in detail previously (1, 3, 4, 6).

[1] The formula for this compound, kindly supplied by Winthrop-Stearns, Inc., is potassium chloride 66.0 per cent, ammonium chloride 12.0 per cent, starch 17.0 per cent, potassium formate 3.0 per cent, calcium formate 1.0 per cent, and magnesium citrate 1.0 per cent.

The daily dietary intake of sodium, potassium, and chloride was omitted for the sake of simplicity but may be determined from Table I as outlined in the experimental methods. The loads of sodium and potassium are indicated in each instance by a circle or square respectively. All values are expressed in milliequivalents per liter and are thus comparable. Dates of venous occlusive operations

TABLE I

Average electrolyte and nitrogen composition of diet given daily to each dog

	K	Na	Cl	N_2
	mEq./kg Wet wt.	*mEq./kg Wet wt.*	*mEq./kg Wet wt.*	*Gms./kg Wet wt.*
Horsemeat	49.4	25.1	13.8	44.6
Low Protein	90.5	54.3	64.7	0.12

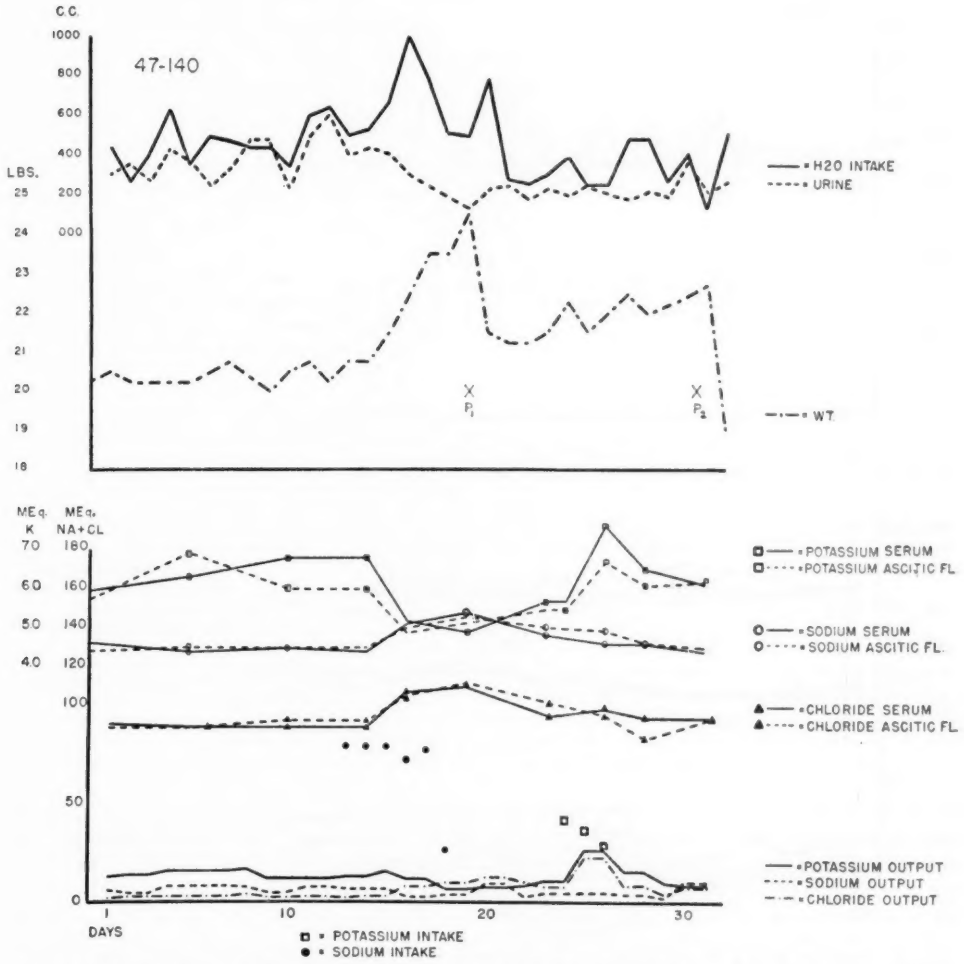

FIG. 1. ELECTROLYTE AND WATER BALANCES OF DOG 47-140, WITH PARTIAL OCCLUSION OF THE INFERIOR VENA CAVA ABOVE THE LIVER PRIOR TO METABOLIC STUDY

Twenty-one grams of sodium chloride (Na) orally during the 13th to 19th days, and 10.5 grams of neocurtasal (K) orally during the 25th to 27th days. Paracenteses (P) of 1850 cc. and 1780 cc. on the 20th and 32nd days.

where they occurred in the course of these experiments are indicated by an "O," and dates of paracenteses are shown by a "P." In two dogs, 47–140 and 50–5, the operation was performed prior to the balance studies shown in the graphs.

Administration of sodium chloride to animals 47–140 (Figure 1) and 50–5 (Figure 2) with partial occlusion of the vena cava above the liver caused a prompt predictable increase in the formation of ascitic fluid, associated with a decrease in the levels of circulating plasma proteins. The amount of sodium in the ascitic fluid recovered by paracentesis was approximately equal to that given orally and it was possible to predict the amount of

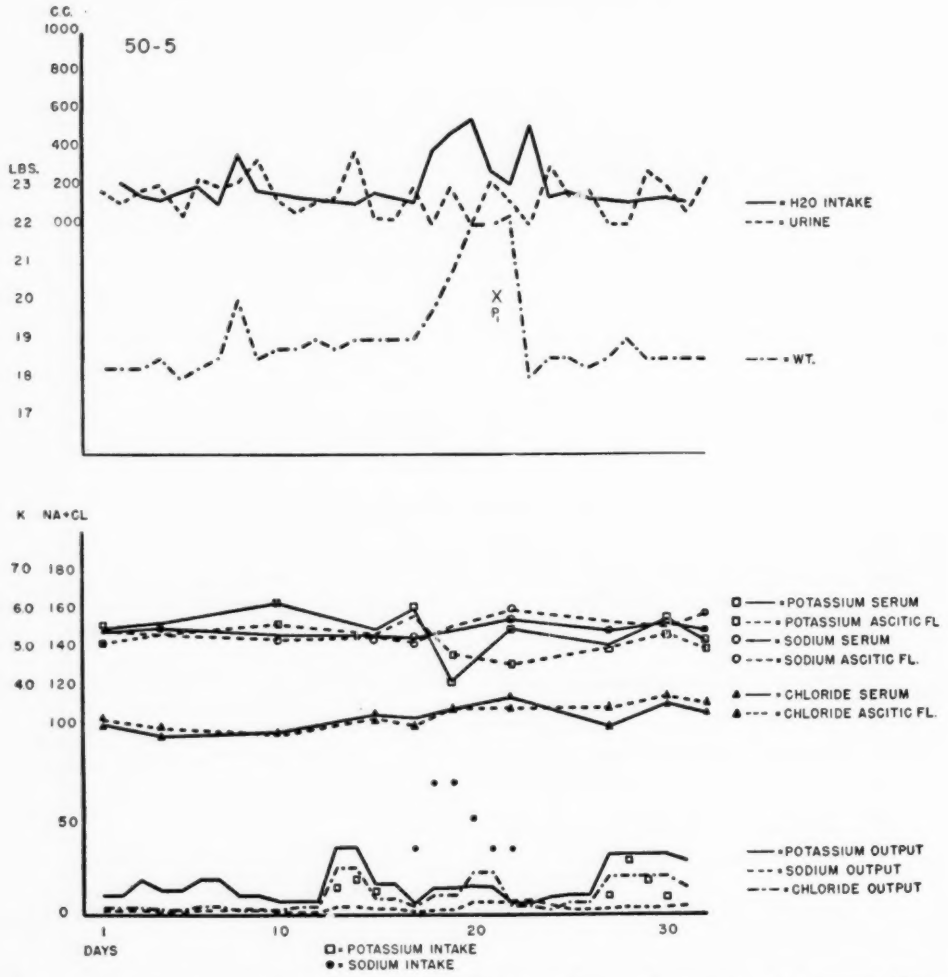

FIG. 2. ELECTROLYTE AND WATER BALANCES OF DOG 50–5, WITH PARTIAL OCCLUSION OF THE INFERIOR VENA CAVA ABOVE THE LIVER PRIOR TO METABOLIC STUDY

4.5 grams of neocurtasal (K) during the 13th to 15th days, 17 grams of sodium chloride (Na) during the 17th to 22nd days, and 6.5 grams of neocurtasal (K) during the 27th to 30th days. Paracentesis (P) of 2230 cc. on the 22nd day.

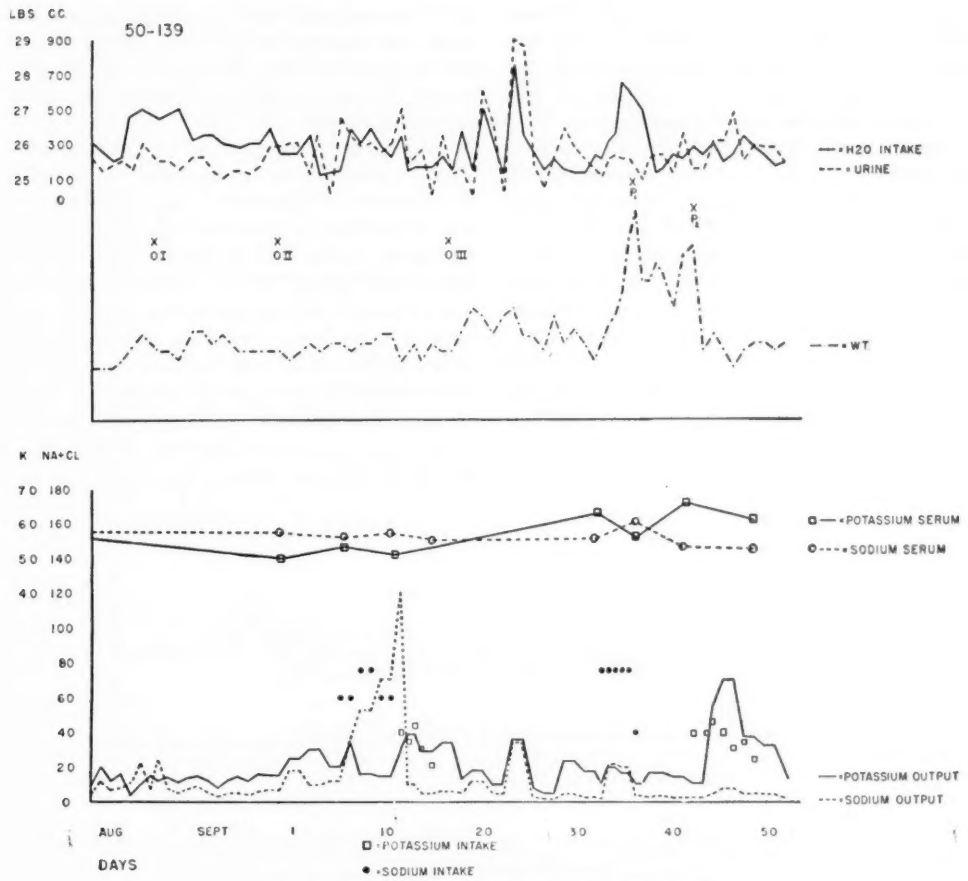

FIG. 3. ELECTROLYTE AND WATER BALANCES OF DOG 50–139

O I and O II represent partial and complete occlusion of the portal vein and the vena cava below the liver and above the kidneys. O III represents partial occlusion of the inferior vena cava above the liver. Nineteen grams of sodium chloride (Na) and 9.5 grams of neocurtasal (K) during the sixth to 11th and 12th to 16th days respectively, and 22 grams of sodium chloride (Na) and 15 grams of neocurtasal (K) during the 33rd to 38th and 45th to 52nd days respectively. Paracenteses (P) of 1200 cc. and 1390 cc. on the 38th and 45th days respectively.

ascitic fluid that would be formed on the basis of the salt intake alone. There was no increase in sodium excretion in the urine of either animal. These observations have been confirmed repeatedly on other animals not used in these metabolic experiments (19). The amount of sodium in the ascitic fluid and urine of dog 47–140 was somewhat greater than the total sodium intake during the period of the experiment, resulting in a net depletion of the total body sodium. Administration of potassium to this animal (47–140) seemed to cause increased ascitic fluid formation presumably by displacement of intracellular sodium into the ascitic fluid. In animal 50–5 potassium was first administered resulting in a prompt, almost quantitative, excretion in the urine with no detectable increase in ascitic fluid formation. Following oral administration of 17 grams of sodium chloride and paracentesis, the loss of sodium in the ascitic fluid and urine during this period almost

exactly equalled the total amount of sodium intake. The third animal, 50–139 (Figure 3), was first studied after a two stage operative procedure that completely divided the portal vein and the vena cava above the kidneys and below the liver. Presumably the mechanical effect of these procedures on the renal circulation was more profound than partial occlusion of the inferior vena cava above the liver in the animals 47–140 and 50–5. Yet these procedures were tolerated with no detectable adverse clinical effect, edema of the extremities, or ascites. Venous pressures were not measured in these animals but in other experiments elevation of inferior caval and portal pressures after partial or complete occlusion decreased to normal ranges quite promptly with the development of collateral circulation through the azygos system (19, 20). With regard to the portal system, Hoffbauer (21) has concluded that levels of pressure in the portal vein could not be correlated with the formation of ascites and that the development of a collateral circulation occurs at relatively low levels of venous pressure. A sodium and potassium load resulted in the urinary excretion of both ions. There was a delay of approximately 36 hours in the diuresis of sodium and a very prompt excretion of potassium. There was some retention following the sodium load during the sixth to 11th days of the balance period. The third operation was performed with partial occlusion of the vena cava above the liver. Following an adequate recovery period a similar salt load was administered. This resulted in the prompt formation of large amounts of ascitic fluid (2590 cc.) with no diuresis of sodium, and the animal was clinically identical to the first two animals; namely, 47–140 and

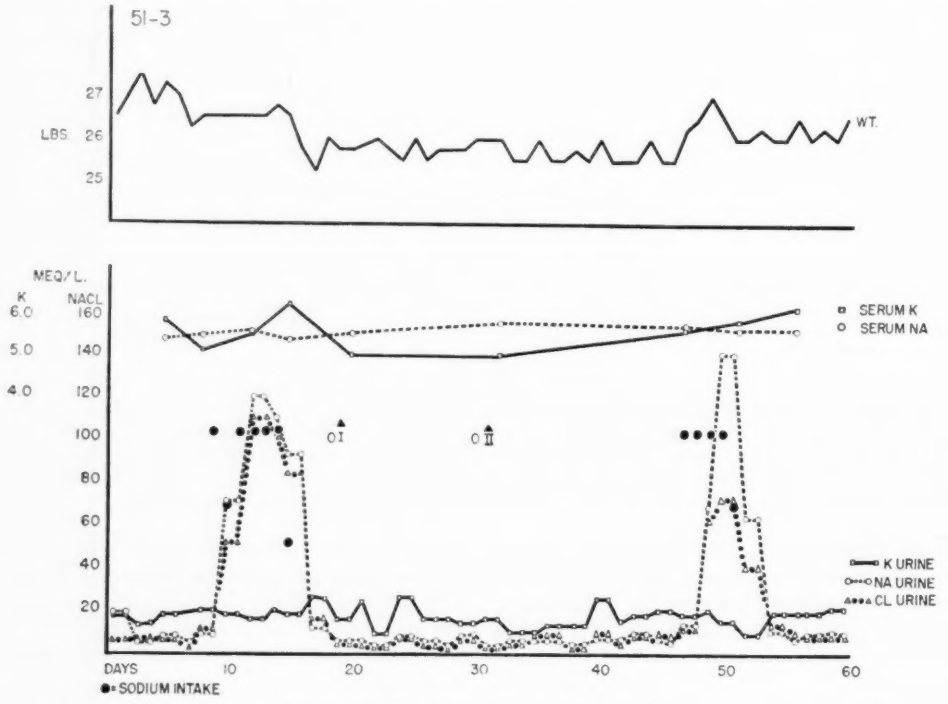

FIG. 4. ELECTROLYTE BALANCES OF DOG 51–3

O I and O II represent partial and complete occlusion of the portal vein and the vena cava below the liver and above the kidneys. Thirty-seven and 28 grams of sodium chloride (Na) were given orally on the ninth to 15th and 47th to 51st days respectively. No ascites.

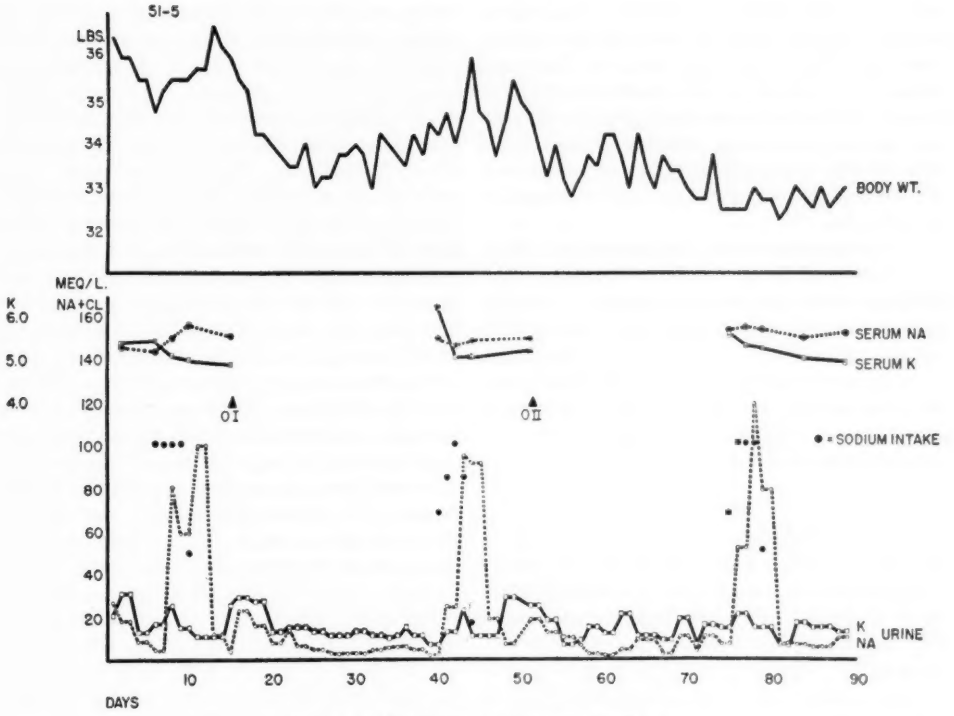

FIG. 5. ELECTROLYTE BALANCES OF DOG 51–5.

O I and O II represent partial and complete occlusion of the portal vein and the vena cava below the liver and above the kidneys. Twenty-seven, 21, and 25 grams of sodium chloride (Na) were given orally on the sixth to tenth, 40th to 44th, and 75th to 79th days respectively. No ascites.

50–5. Similar observations have been made on four other animals with this three stage operative sequence that were not used for metabolic study. Administration of potassium resulted again in prompt excretion with no retention in intracellular fluids.

The fourth and fifth animals, 51–3 (Figure 4) and 51–5 (Figure 5), were observed before and after partial and then complete occlusion of the portal vein and the vena cava below the liver and above the kidneys. Review of the accompanying data reveals no significant retention of sodium. Following partial and complete occlusion of the portal vein and the vena cava, there was a slightly greater delay in the urinary excretion of sodium after excessive administration with temporary retention. There was a small transient increase in the weight of these animals following a salt load, though in no way comparable to the dogs with partial occlusion of the inferior vena cava above the liver and ascites. Ascites has never been observed in our experience in any of our dogs with partial or complete occlusion of the portal vein and the vena cava below the liver.

There was in all animals a very accurate regulation of the serum levels of chloride, sodium, and potassium. The levels of non-protein-nitrogen in the serum were constant and normal. Urea clearances were normal. Sodium, potassium, and chloride were excreted by the kidneys in small constant amounts during the resting periods in the ascitic dogs with a continuous positive balance of 2 to 6 milliequivalents each day. The chloride excretion very closely paralleled the sodium excretion in all animals. In the ascitic dogs there was a remarkable similarity between the serum

and ascitic fluid electrolyte patterns, indicating a prompt and free exchange of water and electrolytes. Previously, McKee and his associates have described the similarity of the electrophoretic patterns of plasma and ascitic fluid proteins (*2*) and their prompt interchange utilizing lysine labeled with C^{14} (*6*). Serum level trends of sodium and potassium have invariably been mirror images of the other ion.

In summary, there was a slightly delayed diuresis of sodium following a salt load with temporary retention in the animals with complete occlusion of the portal vein and the vena cava below the liver and above the kidneys. In the animals with partial occlusion of the inferior vena cava above the liver, there was prompt formation of ascites containing approximately all of the sodium administered, with no increase in urinary excretion.

DISCUSSION

Recent clinical studies of edema and ascites in heart failure and cirrhosis indicate that the kidney is responsible for the retention of sodium that occurs. Our experimental findings in these ascitic dogs suggest that the liver is also involved in this abnormality. Retention of sodium, irrespective of etiology, seems to be the most important factor in the ascitic fluid formation in these animals. The lack of abnormal sodium retention with complete occlusion of the portal vein and the vena cava below the liver and above the kidneys emphasizes the lack of effect of temporary venous stasis alone. Ultimately, dogs cease to produce ascites with partial occlusion of the vena cava above the liver. These animals will again produce ascites for a short period following another operation and complete occlusion of the vena cava above the liver (*19*). Thus, when there is stasis of the hepatic veins in the liver there is a much greater tendency for sodium retention and ascites is formed over a long period. The reasons for this are not clear. Apparently the fluid content of the peritoneal cavity shares to a greater or lesser degree in the expansion and contraction of the interstitial fluid volume, though in an altered way when abnormal hydrostatic, osmotic, or permeability factors exist in the portal system. Rather than ascribe this to a metabolic alteration, it seems simpler, at present, to consider this due to mechanical alterations within the liver parenchyma and related to the unique anatomy of the liver lobule. There the intimate relationships of the lymphatics to the sinusoids may render them more susceptible to minor changes in venous pressure and stasis. Grindlay and his associates (*22, 23*) have demonstrated a marked increase in liver lymph under these circumstances. Thus, the peritoneal cavity may become an anatomical area, with little tissue pressure, trapping sodium and chloride, rather than allowing their participation in a general extracellular water expansion with subsequent renal excretion. Conversely, sodium and water retention may be a renal compensatory mechanism to offset the loss of body fluids, plasma proteins, and electrolytes into the peritoneal cavity. Stamler and his coworkers (*15*) have demonstrated that occlusion of the vena cava above the liver influences renal function indirectly. Here, with the formation of ascites, there is a permanent reduction of renal plasma flow, glomerular filtration rate, and sodium excretory capacity. This change seemed to occur, not in response to the elevated renal venous pressure, but to the over-all changes in the circulation and in the body fluid compartments. Hwang and his associates (*24*) have shown that constriction of the vena cava above the kidneys and below the liver does not cause permanent abnormalities of renal function, though there is temporary reduction in renal plasma flow, renal filtration rate, and sodium excretory capacity. Increased venous pressure *per se* does not cause ascites or a pleural effusion. In none of our animals has ascites been noted following ligation of the portal vein. It is well known clinically that extrahepatic block of the portal system does not cause ascites; that ascites when it occurs with portal hypertension is indicative of intrahepatic fibrosis and damage; and that the surgical prognosis is much more grave when ascites and portal hypertension coexist (*25–27*). In cirrhosis with intrahepatic portal obstruction and ascites, there is no inferior vena cava obstruction. Thus, sodium retention by the kidneys could be a compensatory mechanism from loss of fluid and electrolytes, a primary mechanism initiated by the hepatic elaboration of an antidiuretic substance, or failure of the liver to inactivate an antidiuretic substance produced elsewhere. The pulmonary veins may be constricted to the point of death without pleural effusion (*28*) and the superior vena cava may be tied off with im-

punity, provided the azygos system is intact (29).

Recently the diurnal variation of water and sodium excretion in patients with congestive heart failure, cirrhosis of the liver, and degenerative glomerulo-nephritis, has been emphasized (30). At night there is an increased excretion of water and sodium. This might be related to the increased flow of blood through the liver when the patient is recumbent (31, 32). Much of the clinical and experimental evidence points to an hepatic factor in sodium retention. The exact mechanism of this hepatic abnormality in the renal retention of sodium with ascites formation remains unexplained. Potassium excretion seems to be governed by a different mechanism, corroborating the findings of Baldwin, Kahana and Clarke (33). The liver seems uniquely situated with regard to the effects of venous back pressure, and may elaborate an antidiuretic substance under these circumstances. The adrenals may be implicated, though specific experiments have not been designed to include or exclude them with relation to increased venous pressure.

SUMMARY AND CONCLUSIONS

1. Sodium, potassium, chloride, and water balances were recorded in dogs before and after occlusion of the portal vein and the vena cava below the liver but above the kidneys, and following partial occlusion of the inferior vena cava above the liver.

2. Above the liver, partial occlusion of the inferior vena cava invariably caused retention of sodium and accumulation of ascitic fluid.

3. Administration of excessive amounts of sodium chloride orally caused an immediate and marked accumulation of ascites in all dogs with partial occlusion of the vena cava above the liver. This ascitic fluid contained sodium in amounts approximately equal to the administered excess.

4. Partial and complete occlusion of the portal vein and vena cava below the liver and above the kidneys failed to cause ascites. Excessive administration of sodium in these dogs was unaccompanied by clinical effect, though there was a slightly delayed diuresis of sodium compared to the normal animal.

5. Excessive amounts of potassium (such as neocurtasal) were excreted promptly in all animals with no significant changes in the tissue electrolyte patterns. In certain instances with partial occlusion of the vena cava above the liver, there seemed to be a moderate increase in the ascitic fluid production presumably by displacement of intracellular sodium which was not excreted. Potassium excretion seemed to be governed by mechanisms different from those for sodium.

6. Chloride excretion paralleled sodium excretion in the urine except in instances of high potassium output.

7. Under these experimental conditions chronic congestion of the liver, at least on an anatomical-mechanical basis, seems to be related to this type of ascites and sodium retention.

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RENAL FUNCTION DURING AND AFTER DIABETIC COMA[1]

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Though it has long been clinically recognized that diabetic coma may be associated with renal inefficiency, there is a paucity of quantitative studies of renal function during and after diabetic coma. There are no reports at all in which complete evaluation of renal function by the specific clearance methods of Homer Smith (1) and his coworkers (2) have been attempted. McCance and Widdowson (3) found the glomerular filtration rate lowered in five subjects with diabetic coma. From the low creatinine/inulin clearance ratios which they obtained, these authors believed that tubular injury occurred during coma. It was for the purpose of getting more information on the nature of the renal disturbance in diabetic coma that the present investigation was undertaken. This report deals with the results of serial studies of specific renal function tests in six subjects during diabetic coma and at short intervals thereafter.

METHODS

Clearances of mannitol, p-aminohippurate (PAH), and urea, and measurements of Tm_{PAH} were performed on six patients admitted to the wards of the hospital in diabetic coma. Several clinical features and laboratory findings at the time of hospitalization are presented in Table I. In five subjects the first studies were carried out within five hours of their arrival. Emergency supportive measures had already been started, including insulin and physiological salt solution, but the patients were still comatose (Cases 3, 5, 6) or semicomatose (Cases 2 and 4). Neither the clinical condition nor the emergency management were identical in these cases, but all had comparable hyperglycemia, acidosis, dehydration and electrolyte loss. Tachycardia and hypotension were present in all cases. In no instance was glucose used in the intravenous fluids before or during the clearance tests. In the sixth subject, the renal studies were not begun until the 11th hospital day. However, the routine blood chemical analyses were available from the first day. In addition to the initial renal studies, from one to three tests were carried out later.

For the measurement of glomerular filtration rate (GFR), the mannitol clearance (C_M) was determined. At the time of initiation of these and similar studies, the fact that mannitol clearances were slightly lower than inulin clearances had not been firmly established. The deviations, however, from the true value for GFR are too slight to invalidate the use of mannitol clearances in these studies, for the abnormalities found were significantly large. Each clearance test was performed in four periods, the first two with low serum PAH levels and the last two with high levels for the Tm_{PAH} measurements. In all instances the load of PAH was much greater than was required for saturation of the tubular excretory system. Urea clearances were determined only in the first two periods.

PAH was analyzed by the method described by Goldring and Chasis (2); mannitol, by Corcoran and Page (4); urea, by Hoffman (5). In the mannitol analyses, samples with elevated glucose concentrations were calibrated against standard mannitol solutions to which an equivalent amount of glucose was added. The correction was usually not significant.

RESULTS

The results of the tests for mannitol clearance (C_M), p-aminohippurate clearance (C_{PAH}), urea clearance (C_{Urea}) and Tm_{PAH}, and their ratios, corrected to 1.73 sq. m. of surface area, are shown in Table II. The cases can be divided into two groups according to the pattern of recovery. The data in Group 1, which consisted of Cases 2, 3, 4, and 6, are presented graphically in terms of percentage of normal in Figure 1; those of Group 2, which consisted of Cases 1 and 5, are shown in Figure 2.

The findings in Group 1 were of the same general pattern in all cases. There was, at the time of coma, a reduction to below normal of all clearances. These were restored to normal or supernormal values in a few days after correction of dehydration, acidosis, and electrolyte deficits. Azotemia subsided quickly, the changes of serum nonprotein ni-

[1] Abridgement of material contained in a thesis submitted by Lionel M. Bernstein as partial fulfillment of the requirements for the Degree of Master of Science in Medicine in the Graduate College at the Chicago Professional Colleges of the University of Illinois.

TABLE I

Initial clinical features and laboratory findings

Case	1	2	3	4	5	6
Age	38	27	29	60	22	53
Serum chemical analyses						
NPN, mg./100 cc.	100*	53*	40	104	76	53
Glucose, mg./100 cc.	550*	644	712	985	390	692
CO_2 comb. power, cc./100 cc.	30*	8	10	34	18	8
Na, mEq./liter	—	135	137	146	139	132
Cl, mEq./liter	—	95	101	104	114	100
Urinalyses						
Albumin	4+	0	0	2+	3+	1+
Glucose	4+	4+	4+	3+	4+	4+
Nitroprusside test	4+	4+	4+	1+	4+	4+
Ferric chloride test	4+	4+	4+	0	2+	4+
Specific gravity	1.022†	1.018	1.023	1.030	1.021†	1.025
Blood pressure	105/75	100/70	90/60	82/60	80/55	80/60
Pulse	120	120	132	104	132	120
Respiration	25	40	40	24	40	28
Temperature, rectal, °F.	102.4	102.0	98.6	102.8	99.0	99.0
Precipitating cause of coma	No insulin for 2 weeks	Submandibular abscess; vulvitis	Inadequate insulin therapy (one injection weekly)	Vaginal moniliasis; unknown diabetic	No insulin for 4 weeks. Alcoholic spree	Unknown untreated diabetic

* Serum analyses after 18 hours.
** Isosthenuric urine occurred within a few hours, and persisted for several days thereafter.

trogen levels within 48 hours being 53 to 34, 40 to 20, 100 to 53, and 53 to 45 mg./100 cc. respectively. In contrast with these patients, those of Group 2 showed an azotemia which became more intense in spite of correction of the several deficits. The uremia, however, was of limited duration.

TABLE II

*Specific renal function**

Case	Surface area	Hospital day	C_M	C_{PAH}	Tm_{PAH}	$C_{U_{res}}$	C_M/C_{PAH}	C_{PAH}/Tm_{PAH}	C_M/Tm_{PAH}
			cc./min.	*cc./min.*	*mg./min.*	*cc./min.*			
1. G.H.	1.55	11	21.6	77.4	5.2	16.9	0.28	15.0	4.2
		21	62.9	519.0	56.5	50.7	0.12	9.2	1.1
		36	70.0	454.0	68.2	48.0	0.15	6.6	1.0
2. A.L.	1.4	1	98.0	381.0	37.4	48.8	0.26	10.2	2.62
		9	128.0	725.0	104.0	76.9	0.18	7.0	1.23
3. A.H.	1.68	1	95.0	398.0	49.7	36.2	0.24	8.0	1.9
		13	170.0	902.0	111.0	101.0	0.19	8.2	1.53
		21	172.0	816.0	142.0	77.0	0.21	5.8	1.21
4. L.R.	1.42	1	13.2	148.0	71.5	10.3	0.09	2.1	0.19
		8	99.0	488.0	79.5	68.2	0.20	6.2	1.25
5. J.C.	1.66	1	0.0	0.0	0.0	0.0	—	—	—
		6	11.5	25.3	2.53	5.95	0.46	10.0	4.5
		15	66.0	303.0	61.7	44.2	0.22	4.9	1.1
		21	85.2	577.0	93.5	56.2	0.15	6.2	0.91
6. M.R.	1.8	1	24.2	162.0	28.2	14.0	0.15	5.8	0.86
		12	75.2	580.0	89.7	49.5	0.13	6.5	0.85
		36	94.0	595.0	114.0	40.2	0.16	5.2	0.83
Normal Values			117±15.6	594±102	77.5±12.9	75±15	0.20	7.6	1.51

* Corrected to 1.73 sq. m. of surface area.

The serum nonprotein nitrogen levels, which reached peaks of 158 and 122 mg./100 cc. respectively, returned to normal within two weeks (see Figure 2). The serum creatinine concentrations in these two cases reached levels of 8 mg./100 cc., which is much higher than is seen in simple dehydration. C_M, C_{PAH}, and Tm_{PAH} were markedly reduced in the initial tests, and the ratios were decidedly abnormal. Only slowly was there evidence of a return to normal function. As will be discussed later, it is probable that in these two patients a reversible organic lesion had occurred.

In all cases, the return to essentially normal values of renal function excluded the possibility that the initial renal disturbances were due to intrinsic renal disease not related to the diabetic acidosis.

DISCUSSION

Much of the interpretation of the clearance values and their ratios found in these studies depends upon the significance of C_{PAH} and Tm_{PAH}. If it can be assumed that, in the cases of Group 1, the extraction of PAH was not appreciably reduced by the disease, C_{PAH} can be regarded as a measure of effective renal plasma flow, and Tm_{PAH} as a measure of maximal tubular excretory capacity. Several features of the data point to the validity of such an assumption. In the first place, Tm_{PAH} was nearly normal in Case 4 during the coma period, even when C_M, C_{Urea}, and C_{PAH} were greatly reduced. Thus a normal PAH extraction is at least possible in the presence of severe diabetic acidosis. Secondly the ratios C_M/C_{PAH}, C_{PAH}/Tm_{PAH}, and C_M/Tm_{PAH} were not unreasonably deviated from normal in any of the four cases of Group 1. If the tubular extraction of PAH were poor, the ratios C_M/C_{PAH} and C_M/Tm_{PAH} should have been unusually high, unless an independent reduction in C_M equal to the reduction in extraction of PAH had occurred. On the other hand it is theoretically plausible that the combination of dehydration, renal ischemia, cellular deficiency of potassium, phosphorus, and other constituents, as well as disturbances in endocrine secretions, would markedly alter the ability of the tubular cells to excrete PAH from the renal interstitial fluid. The recent findings (6), that the amount of available acetate and possibly other metabolic intermediates influences the tubular excretory capacity, serve to emphasize the dependence of tubular function upon normal cellular metabolism.

If the extraction of PAH was subnormal in the initial studies on the patients of Group 1, the data

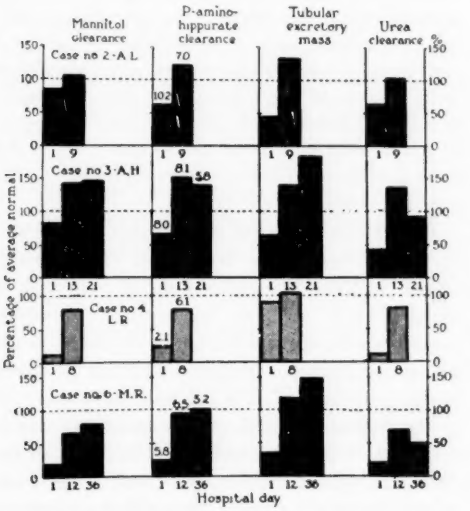

FIG. 1. SPECIFIC RENAL FUNCTION TESTS OF GROUP 1

Values are presented as per cent of average normal after correction for surface area. The numbers above the p-aminohippurate bars are the ratios of C_{PAH} to Tm_{PAH}.

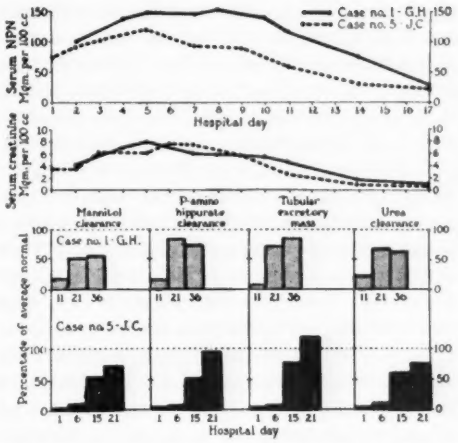

FIG. 2. SERIAL SERUM NPN AND CREATININE VALUES, AND THE SPECIFIC RENAL FUNCTION TESTS OF GROUP 2

Renal function tests are presented as per cent of average normal after correction for surface area.

defy interpretation, other than the generalization that both glomerular and tubular functions were depressed during diabetic coma and that they were quickly restored to normal or better than normal. If, however, the assumption, based on the above mentioned considerations, is made that the impairment of extraction of PAH was not great enough to alter the validity of the usual interpretations of clearance data, then a reasonable analysis of the events is possible. In Cases 2 and 3, the effective renal plasma flows were considerably reduced while the glomerular filtration rates were nearly normal. The filtration fractions were elevated. At the same time, Tm_{PAH} was markedly lowered. These findings implied a virtual exclusion from functioning, at least momentarily, of a large number of nephrons, but a normal or supernormal blood supply to those which were functioning. On the other hand, in Case 4 a normal Tm_{PAH} was associated with greatly depressed values for C_M and C_{PAH} and C_{Urea}. The ratio C_{PAH}/Tm_{PAH} was 2.1 instead of the normal 7.6, which finding implied severe ischemia of all nephrons. This diminished renal blood flow was undoubtedly related to a decreased cardiac output. The fact that the filtration fraction was also very low—0.09 instead of the normal 0.20—indicated that any renal arteriolar constriction involved in the renal ischemia failed to produce a rise in effective intraglomerular pressure. The presence of fever in this patient may account for the low filtration fraction by the production of a relative efferent arteriolar dilatation. The low filtration rate in the presence of normal Tm permits a reasonable explanation for the unusual (but not rare) findings of moderate glycosuria in spite of intense hyperglycemia, and of an almost complete absence of acetoacetic acid in the urine (see Table I). That the renal ischemia was prolonged and severe but still reversible is seen in the serum nonprotein nitrogen concentration of 100 mg./100 cc., and its fall to nearly normal in 48 hours. In Case 6, too, the data point to ischemia of the working nephrons, with many nephrons showing virtually no function, since Tm_{PAH} was reduced to a third of normal.

It is worthy of note that following recovery from the diabetic coma, the patients in Group 1 showed not merely a rapid return of Tm_{PAH} to normal levels, but actually an elevation to supernormal values (Cases 2, 3, and 6). That this occurrence of supernormal values is not an isolated finding peculiar to diabetes is seen from reports of similarly high Tm_{PAH} values under other disease or experimental conditions (7–10). Sirota (9), for example, reported a Tm_{PAH} of 168 per cent of normal (130 mg./min.) following recovery from "lower nephron nephrosis" due to carbon tetrachloride poisoning. What the significance of these supernormal findings in these different situations can be is a matter of conjecture.

In the two patients in Group 2 there need be less conjecture about the completeness of extraction of PAH by the tubules. The extremely low Tm_{PAH} (6.7 per cent of normal on the 11th day in Case 1 and 3.3 per cent of normal on the sixth day in Case 5) and the distorted, unreasonable ratios point to poor extraction of PAH. This phenomenon might be due to injury to proximal tubules caused by a poor renal blood flow. But if this were the nature of the injury, the glomerular filtration rate and serum nonprotein nitrogen concentration should have returned to normal by the time of these tests, for dehydration, ketosis, and electrolyte deficits had already been corrected. It was more likely that the poor extraction, the exaggerated ratios, the relatively prolonged azotemia, the isosthenuria, and the slow return of the renal function tests to normal values were associated with damage to the tubules and with interstitial edema. In other words these patients presented, in a relatively mild form, the picture seen in the type of acute renal failure commonly called "lower nephron nephrosis" (9, 11, 12).

Only in the absence of recognizable oliguria and in the mildness of the uremia did the syndrome shown by these two patients differ from that of usual "lower nephron nephrosis." However, though severe oliguria bordering on anuria is the presenting symptom in almost all cases of "lower nephron nephrosis," it is not necessarily essential to the disease. In such cases, oliguria appears to be due to a large nonspecific reabsorption of tubular fluid across the damaged tubular membrane in relation to the amount of modified glomerular filtrate presented to it. If the glomerular filtration is relatively large, the quantity of fluid un-

reabsorbed and ultimately excreted as an isosthenuric urine may be considerable. Indeed this phenomenon is what probably occurs during the first days of diuresis in all cases of "lower nephron nephrosis." In spite of the absence of oliguria, the nitrogenous excretion may be low enough to produce prolonged azotemia. One of Sirota's cases of carbon tetrachloride poisoning (9) had no demonstrable period of oliguria, and yet had a C_I of 3.78 cc./min., C_{PAH} of 14.4 cc./min., and Tm_{PAH} of 1.85 on the tenth day at a time when the urine output was 2290 cc./day. Burnett, Burrows and Commons (13) intimate such a phenomenon in their cases of alkalosis. We have seen comparable cases of alkalosis of pyloric obstruction with protracted uremia but with eventual return of normal function. The transient uremia without oliguria following diabetic coma reported by McCance and Lawrence (14) may be of the same type.

The alterations in renal function in the cases of both Groups 1 and 2 may be consequences of dehydration of varying degrees of severity. Depletion of body water and electrolytes results in decreased blood volume and diminished cardiac output causing a reduction of renal blood flow as part of a reduction of flow to all areas of the periphery. Renal hemodynamic changes secondary to hypotension accentuate the diminution in renal blood flow. The changes may involve all nephrons; or many nephrons may be temporarily deprived of function, the remaining nephrons having low, normal, or supernormal blood supply. If the ischemia and metabolic insult is not severe, the alterations in renal function are rapidly reversed by correction of the deficits of water, electrolytes, and insulin, as in the cases of Group 1. When the process is more severe, acute organic damage to the distal tubules may develop leading to "lower nephron nephrosis," as in Group 2.

SUMMARY

The specific clearance methods of Homer Smith were utilized for the study of renal function during and after diabetic coma. The six cases could be divided into two groups.

In the four patients of Group 1, there was, at the time of coma, a reduction of C_M, C_{PAH}, and Tm_{PAH}. These were quickly restored to normal or supernormal values after repair of dehydration and electrolyte deficits. These rapidly reversed alterations of renal function were believed to be caused by dehydration with its accompanying decrease in blood volume, cardiac output, and renal blood flow, and by the exaggeration of the last defect by the renal hemodynamic response to such dehydration.

In contrast, the two patients in Group 2 showed azotemia which progressed in spite of correction of water and electrolyte loss, and which lasted two weeks. Several days after recovery from coma, C_M, C_{PAH}, and Tm_{PAH} were still markedly reduced, and the ratios were distorted. Only slowly did these values return to normal. In these patients the renal ischemia had apparently produced a reversible organic lesion similar to that found in "lower nephron nephrosis," except that it was milder and that it was unaccompanied by initial oliguria.

ACKNOWLEDGMENTS

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EFFECT ON FEMORAL A-V GLUCOSE DIFFERENCE OF INSULIN INJECTED INTO AN ANTECUBITAL VEIN AND INTO A FEMORAL ARTERY [1]

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There still exists considerable controversy with respect to the importance of the liver as compared to the peripheral tissues (skeletal muscle) in the homeostasis of blood sugar levels. The place and mechanism of action of insulin remain unclear.

Mann and Magath (1) are credited with first establishing the peripheral action of insulin apart from the liver. This was done in evisceration experiments in dogs. Frank, Nothmann and Wagner (2, 3), working with rabbits and pancreatectomized dogs, claimed a local peripheral action of insulin following its injection into a femoral artery. *In vitro,* it has been shown that the glucose uptake of the rat diaphragm is increased under the action of added insulin (4–6). Stadie and Zapp (7) have demonstrated that insulin is fixed to the diaphragm and cannot be washed off.

The purpose of this paper is to record data demonstrating the different effect on femoral A–V glucose difference of insulin injected into a femoral artery and into an antecubital vein.

METHODS

Convalescent hospital patients and normal medical students served as subjects.

All studies were done in the morning and the subjects had had no food for 12 to 14 hours. They were at rest for an hour prior to insulin injection. No attempt was made to control room temperature.

The main femoral artery and vein were used for sampling. Samples were drawn simultaneously into heparinized syringes and immediately transferred to tubes prepared by the evaporation of 1 ml. of 3 percent sodium fluoride and these were then placed in ice. Using barium hydroxide and zinc sulfate precipitation, filtrates were made at the completion of the experiment.

Crystalline Zinc Insulin (Squibb and Lilly), freshly diluted in saline for each experiment, was used. In all cases, the dose was 1/80 unit per kilogram. The time of injection was one-half minute or less, unless otherwise stated.

The blood sugar concentrations were determined on 2 ml. of whole blood by the Iodometric Titration method of Somogyi (8, 9). In our hands this method provided a high degree of accuracy. The average standard deviation calculated from results obtained from replicate samples of whole blood was ± 0.47 mg. per 100 ml. A–V glucose differences of 2 mg. per 100 ml. or greater are significant. The resting arterial, venous and A-V glucose concentration was found to be remarkably constant in a series of 22 subjects when checked over intervals of two to 25 minutes (10).

Blood oxygen concentration was determined by the method of Hickam and Frayser (11).

RESULTS

The first set of observations was made on hospital patients who were considered free from metabolic disorders and were well-nourished. They were divided into two groups of ten patients each. Insulin was injected intravenously into an antecubital vein in one group and intra-arterially into the right femoral artery in the remainder. In all cases, blood was obtained from the main fe-

TABLE I

Femoral arterio-venous blood sugar differences
(mg. per 100 ml.)
Hospital patients receiving 1/80 unit insulin per kilogram into antecubital vein

Pt.	Control	5 min.	15 min.	30 min.	45 min.	60 min.
J. A.	4.5	3.9	2.4	5.0	13.8	
S. S.	3.8	0.7	−3.1	1.8	3.2	
N.	−0.5	−0.1	−1.4	2.4		10.9
R. S.	−0.4	1.9	0.4	2.0		
J.	3.7	1.2	0.1	4.2		9.0
W. S.	6.2	−0.7	−1.5			
R.	5.5	3.7	0.1	6.8	4.7	
J. J.	5.5	−2.1	3.7	4.3		
M. P.	2.6	1.1	−3.1	2.5	−0.6	0.6
M. P.	3.2	−1.5	0.1	2.3		3.1
MEAN	3.4	0.8 p<0.01	−0.23 p<0.01	3.4	5.2	5.7

p—refers to A-V differences at the times listed, compared to control values.

[1] This investigation was supported in part by a research grant from the Life Insurance Medical Research Fund and in part by a research grant from the National Institutes of Health, U. S. Public Health Service.

TABLE II

Femoral arterio-venous blood sugar differences
(mg. per 100 ml.)
Hospital patients receiving 1/80 unit insulin per kilogram into right femoral artery

Pt.	Control	5 min.	15 min.	30 min.	45 min.	60 min.
W. T.	1.0	2.7	1.0	5.0	5.8	4.2
N. B.	0.7	2.0	3.8	2.8	1.1	3.3
L. W.	1.7	7.4	5.1	5.6	9.2	
J. N.	−0.9	4.9	1.8	14.8	19.5	15.8
B.	−0.5	0.6	4.0	6.9		6.9
S.	0.9			14.8		18.6
E. L.	4.3	3.7	4.0	7.4	4.1	
M. S.	0.6		2.7	5.7		
B.	6.0	6.8	6.7	7.4		6.6
C.		−0.6	2.3			5.2
MEAN	1.5	3.4 $p<0.2$	3.4 $p<0.1$	7.8 $p<0.01$	7.9 $p<0.05$	8.6

p—refers to A-V differences at the times listed, compared to control values.

moral artery and vein of the right leg. Table I presents the A-V sugar differences at various times after the intravenous injection of insulin. During the period of fall in arterial sugar there was not a measurable A-V difference, but following the 15-minute period when the arterial concentration was rising, a measurable A-V glucose difference returned. Table II presents the data on the second group of hospital patients in whom the insulin was injected intra-arterially. The A-V sugar differences are statistically wider at 15 and 30 minutes than those after intravenous injection ($p = 0.01$ and $p < 0.01$). Figures 1 and 2 are composite curves constructed from the average value in milligrams percent at each time interval.

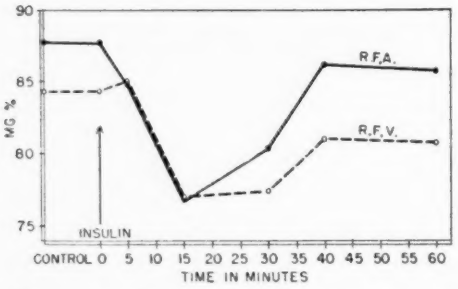

FIG. 1. COMPOSITE GRAPH: TEN PATIENTS WHO RECEIVED INTRAVENOUS INSULIN (1/80 UNIT PER KILOGRAM)

R.F.A.—Glucose concentration in right femoral artery
R.F.V.—Glucose concentration in right femoral vein

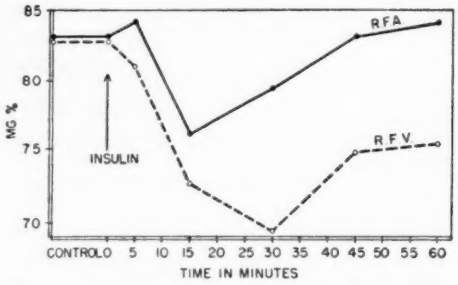

FIG. 2. COMPOSITE GRAPH: TEN PATIENTS WHO RECEIVED INTRA-ARTERIAL INSULIN (1/80 UNIT PER KILOGRAM)

R.F.A.—Glucose concentration in right femoral artery
R.F.V.—Glucose concentration in right femoral vein

The second set of observations was made on seven normal male students. Insulin was injected intra-arterially into the right femoral artery, and venous blood was obtained from the main femoral veins of both legs. Table III and Figure 3 present the A-V differences in blood sugar as found in this group. It should be noted that the last two subjects in Table III (K. G. and B. N.) received insulin given over a five-minute period. During

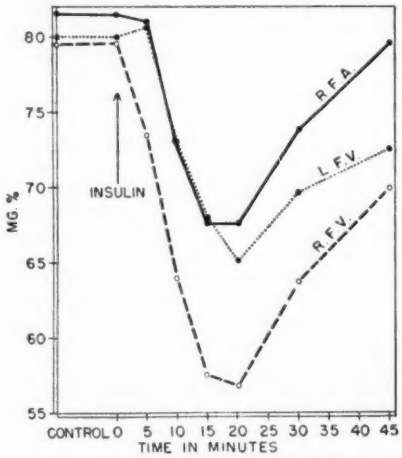

FIG. 3. COMPOSITE GRAPH: SEVEN NORMAL STUDENTS WHO RECEIVED INTRA-ARTERIAL INSULIN (1/80 UNIT PER KILOGRAM)

R.F.A.—Glucose concentration in right femoral artery
R.F.V.—Glucose concentration in right femoral vein
L.F.V.—Glucose concentration in left femoral vein

TABLE III

Femoral arterio-venous blood sugar differences (mg. per 100 ml.) in normal students receiving 1/80 unit insulin per kilogram into right femoral artery

Subj.	Control		5 min.		10 min.		15 min.		20 min.		30 min.		45 min.	
	RFV	LFV	RFV	LFV	RFV	LFV	RFV	LFV	RFV	LFV	RFV	LFV	RFV	LFV
D. B.	1.6	1.2						−8.1	13.8	2.3	10.7	−2.8	16.0	14.4
J. W.	0	−1.8					4.4	−5.7	4.5	−2.3	9.0	7.6	8.5	4.9
K. W.	1.7	1.1					12.5	3.6	12.6	3.9	4.9	1.6	4.2	1.7
J. H.	2.6	1.2	2.9	2.7	4.0	0.4	3.0	0	8.1	5.4	5.6	3.8		
S. V.	3.6	2.0			9.6	1.1	8.2	0	9.2	3.9	11.0	6.6		
K. G.	1.4	1.9	12.2	−0.8	16.4	−0.9	18.2	1.2	19.4	3.3	18.8	7.3		
B. N.	3.9	4.1	8.4	−2.7	6.5	−0.5	11.3	6.1	8.0	0.8	10.1	4.8		
MEAN	2.1	1.4	7.6	0.4	9.1	0.02	9.6	−0.4	10.8	2.5	10.0	4.1	9.6	7.0
			$p<0.02$ $p'<0.01$		$p<0.02$ $p'<0.01$		$p<0.01$ $p'<0.01$		$p<0.01$ $p'<0.01$		$p<0.05$ $p'<0.01$			

RFV—Arterio-venous difference in right femoral artery and right femoral vein.
LFV—Arterio-venous difference in right femoral artery and left femoral vein.
p—Comparing RFV and LFV at each time.
p′—Comparing RFV at each time with control RFV.

the fall in arterial glucose concentration, all subjects had a greater A-V sugar difference across the leg into which the insulin was directed. In the opposite leg, the A-V glucose difference disappeared during the period of fall in arterial glucose concentration.

Table IV presents the oxygen values obtained on the student group. The exact time of sampling varied from subject to subject and the time grouping in this table is approximate. These data show no consistent trend and are presented to show that the observed changes in A-V sugar differences in the injected leg were not caused by a striking reduction in blood flow. This is based on the assumption that the increased removal of sugar after insulin does not cause a major change in oxygen uptake (4–6), and therefore the A-V oxygen difference can be used to determine directional changes in blood flow.

TABLE IV

Femoral arterio-venous blood oxygen differences
(Vol. percent)
Normal student group—insulin in right femoral artery

	Control		15 min.		22 min.		40 min.	
	RFV	LFV	RFV	LFV	RFV	LFV	RFV	LFV
D. B.			7.3				5.3	
J. W.	3.5				2.9			
K. W.					1.9	4.4	4.0	3.6
J. H.	3.3	3.1			3.0	3.1	3.6	3.4
S. V.	3.9	4.7			4.8	5.4	4.8	5.5
K. G.	6.6	6.7	9.5	8.8	6.4	6.6	7.3	7.3
B. N.	3.9	5.2			4.8	6.5	4.8	6.2
MEAN	4.2	4.9	8.4	8.8	4.0	5.2	5.0	5.2

RFV—Arterio-venous difference right femoral artery and right femoral vein.
LFV—Arterio-venous difference right femoral artery and left femoral vein.

DISCUSSION

Study of the sites of action of insulin in the body is complicated by the fact that any large change in the blood sugar sets in motion a series of homeostatic reactions. The problem is simplified by using a dose of insulin just large enough to cause a definite but small lowering of the arterial blood sugar level.

The intravenous injection of 1/80 unit of insulin in the hospital patients caused an average fall of 11 mg. per 100 ml. in the arterial blood sugar level. From inspection of the arterial and femoral venous curves, we cannot tell in what tissue the sugar disappears. The disappearance of the preinjection A-V glucose difference across the leg suggests that in this dose the insulin decreases the rate of removal of sugar by the leg. We do not believe that this means that insulin is interfering with glucose removal, but that the apparent decrease in rate of glucose removal is probably secondary to the fall in the arterial glucose level and probably does not represent a direct effect of insulin on the tissues of the extremity.

In the group of hospital patients receiving insulin intra-arterially, the A-V glucose difference

widened as the arterial blood sugar level fell. The increased A-V glucose difference is significant at 15 and 30 minutes when compared with the slightly negative A-V differences of the intravenous injection.

In the students, the A-V glucose difference widened significantly in the injected leg as the arterial blood sugar level fell. In these healthy students, the action of insulin was greater than in the hospital patients. The wide A-V glucose difference in the injected extremity indicated increased removal of glucose as compared to the opposite extremity. In the non-injected extremity the A-V glucose difference disappeared. Again, this is interpreted as the effect of lowering of arterial glucose concentration.

These data indicate that a large portion of the insulin injected into a femoral artery was active in the extremity and that it increased the glucose removal from blood perfusing that extremity without a corresponding increase in the opposite extremity. During the first 15 minutes, one has the opportunity to observe the effect of a marked increase in insulin activity in one extremity at a time when the complicating effects of the homeostatic mechanisms for restoring the arterial blood sugar level are reduced to a minimum. The concentration of the insulin in the extremity after intra-arterial injection is much greater than after giving the same amount in the antecubital vein. The data presented do not allow us to say whether or not the insulin is fixed in the leg in the same way that it is fixed to the rat's diaphragm.

While the data after intra-arterial injection demonstrate a local peripheral effect of insulin on the rate of glucose removal, the data from the intravenous injection suggest that there are areas in the body that are more sensitive to the action of insulin than the lower extremities.

SUMMARY

1. The injection of 1/80 unit of insulin per kilogram into an antecubital vein caused a decrease in the femoral A-V glucose difference.

2. The injection of 1/80 unit of insulin per kilogram into the right femoral artery caused an increase in the A-V glucose difference in the injected extremity and a decrease in the opposite extremity.

3. The data indicate that the removal of glucose in the leg can be increased by the action of insulin; but when insulin is given intravenously in doses of 1/80 unit per kilogram, the lowering of the arterial sugar is caused by the action of insulin on other tissues more sensitive to it than the lower extremity.

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THE ROLE OF THE GRANULOCYTE AS A SOURCE OF LYSOZYME IN ULCERATIVE COLITIS [1]

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The presence of high concentrations of lysozyme in the stools of patients with ulcerative colitis has been demonstrated by a number of observers. It has been suggested that this enzyme may play a fundamental role in causing destruction of the colonic mucosa. Recent observations, showing that a high concentration of lysozyme is present also in granulation tissue and in leukocytes, have cast doubt on this hypothesis and suggest that the large amount of the enzyme in the feces of patients with ulcerative colitis is a result rather than a cause of the inflammatory process.

The present report summarizes a series of studies designed to elucidate the source of lysozyme in the stools in ulcerative colitis. Our observations lead us to believe that the enzyme content of the feces is derived predominantly from the white blood cells present in the intestinal wall and stools in this disease.

REVIEW OF LITERATURE

In 1922 Fleming (1) drew attention to a powerful bacteriolytic agent, which he named "Lysozyme," present in many body tissues and secretions of man and animals. He noted the high levels of this enzyme in tears, nasal secretion, egg white and leukocytes. Since then, many investigators have helped to clarify the nature of this enzyme, and it is now known to be a protein of low molecular weight capable of depolymerizing certain muco-polysaccharides. Meyer and Hahnel (2) contributed much toward the fundamental knowledge of this enzyme and devised an excellent viscosimetric technique for its quantitation in tissues and secretions, using as a substrate a mucopolysaccharide obtained from the micrococcus lysodeikticus.

The original hope of the early investigators that lysozyme might be used as a therapeutic agent was dispelled by the finding that lysozyme had very little lytic action against pathogens (3). In 1948, Meyer and his associates (4) noted an increased amount of lysozyme in the stools of patients with intestinal ulcerative disease, *i.e.*, regional ileitis and ulcerative colitis. They suspected that lysozyme played a primary role in the etiology of these diseases by removing the protective mucous lining of the gut, thus subjecting the mucous membrane to bacterial and/or enzymatic damage. Initial studies of instilling lysozyme into the intact or isolated portions of the gut of a dog, resulting in superficial ulcerations, seemed to confirm this point of view. However, the amount of lysozyme used was very large, and subsequent investigators, although confirming these observations, disagreed with the interpretation and extent of pathology (5). Also, it appeared unlikely that lysozyme had any effect on the mucus in the gut since the mucus could not be depolymerized *in vitro* (6).

It has been demonstrated conclusively by recent investigations that the titre of fecal lysozyme in patients with ulcerative colitis closely parallels the clinical course, in that the lysozyme titre rises with exacerbations and falls with remissions. Gray, Reifenstein, Benson, and Young (7) carefully recorded the fecal lysozyme titres of patients with ulcerative colitis in whom remissions were precipitated by ACTH, and clearly demonstrated the parallelism between the lysozyme titres and the clinical status of the patient. This relationship was found to be true of patients undergoing spontaneous remissions, as observed during the past three years in the Combined Ulcerative Colitis Clinic of the Columbia-Presbyterian Medical Center. When lysozyme inhibitors were given by mouth to patients with ulcerative colitis, as was done by Gray and associates, who used Aerosol (di-octyl sodium sulfo succinate) (8), the fe-

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cal lysozyme titre fell to low levels without appreciably altering the course of the disease. These observations, plus the fact that lysozyme is found in high quantities in all granulation tissue (9), have led to the belief that lysozyme has no primary role in the etiology of ulcerative colitis, but that it is an accurate index of the degree of inflammatory involvement of the colon.

The fact that lysozyme is present in exudate and granulation tissue was known by most investigators of lysozyme (9, 10); however, the importance of this in ulcerative colitis was largely overlooked. In 1950, Sammons (10) suggested that "pus cells" were responsible for some of the lysozyme content of the stools of patients with ulcerative colitis, but he offered no clear-cut proof. Also in 1950 Gottschall, Pickering and Tabachnik (11), working in this laboratory, demonstrated clearly that lysozyme was to be found only in the granulocyte series (polymorphonuclear leukocytes, eosinophils in circulating blood and exudate) and again drew attention to the large numbers of granulocytes found in the stools of ulcerative colitis patients.

PRESENT STUDIES

We have attempted to correlate the number of granulocytes seen in microscopic examination of the stools in ulcerative colitis with the lysozyme titres, but no accurate correlation was possible because of cellular breakdown, making cell types unrecognizable. Therefore, we resorted to obtaining the specimens from the wall of the rectum by scraping with a blunt scoop during proctoscopic examination. When this material is stained by Wright's method, the cells are more recognizable and a rough correlation can be made between the number of granulocytes and lysozyme titre.

An observation not heretofore recorded was the high lysozyme titre found in the lining mucus of patients in complete clinical remissions from ulcerative colitis. Seventeen such determinations in 17 patients ranged from 69 to 310 units per cubic milliliter, with an average of 150. One patient in remission while still receiving cortisone had a lysozyme titre of 150 units per cubic milliliter. In another patient with a rectal polyp and a normal colon, no lysozyme was found in the mucus removed from the rectal wall and no granulocytes were seen on microscopic examination. Microscopic examination of all specimens removed from patients with ulcerative colitis in remission uniformly revealed vast numbers of granulocytes.

However, the correlation of granulocytes and lysozyme does not completely answer the fundamental question of lysozyme source, since it is theoretically possible that the mucous membrane of the colon secretes lysozyme as a result of the same underlying mechanism that elicits a leukocytic response. The correlation of granulocytes and lysozyme also has the objectionable feature of being subject to interpretive error.

A new method was adopted in an attempt to overcome these deficiencies. It had been observed microscopically that most of the white cells in the material obtained by scraping the rectal mucous membrane were imbedded in the mucus from which they could not be separated by physical means. Shaking this combination of mucus and exudate with saline, centrifuging and determining the lysozyme content of the supernatant fluid always yielded negative results, while the residue of mucus and cells was always quite high in lysozyme content. In order to determine the exact source of lysozyme, the mucus had to be separated from the cells, and to do this, a supply of mucinase which had been extracted from the *Vibrio cholera* was used. When this was first added to a saline suspension of mucus and cells obtained from a patient with ulcerative colitis and then incubated, the mucus lost its physical property of tenacity and could no longer be identified grossly. After centrifuging this specimen, it was found that for the first time, lysozyme appeared in the supernatant fluid as well as in the cellular residue, sometimes in almost equal amounts. The lysozyme in the supernatant portion could be from only two possible sources, the mucus itself or lysozyme left in active form after dissolution of the white cells. The exact source of lysozyme was not determined until a patient with an isolated loop of large intestine, which was completely detached from the fecal stream but in which there was active ulcerative colitis, was studied. It was possible to instill fluid in the proximal end of this isolated loop and obtain it from the distal end.

Theoretically, if the total source of the lysozyme were the invading granulocyte which leaves lysozyme in active form after dissolution of the cell

body, the first washing of the gut loop after a long period of rest should show a significant titre of free lysozyme, which would appear in the supernatant fluid after depolymerization of the mucus and centrifugation. With a short period of rest, the lysozyme should be found only with the intact granulocytes in the residue of the centrifuged and depolymerized specimen.

Confirmation of this theoretical possibility was determined as follows:

Method

The isolated loop of colon of the patient mentioned above, after a 24-hour period of rest, was irrigated with 40 ml. of normal saline (Table I, Specimen A). A second time, ten minutes later, the loop was again irrigated (Specimen B), and a third irrigation was done after another ten minutes (Specimen C). Each specimen was mixed vigorously and a 6 ml. aliquot taken. Mucinase powder was added to each aliquot and incubated until the solution was homogeneous and no more mucus could be demonstrated on the stirring rod. Each specimen was centrifuged for five minutes at high speed and 1 ml. of supernatant fluid removed for microscopic study and lysozyme determination. A microscopic study and lysozyme titre was then done on the residue of each specimen after discarding the remaining supernatant fluid.

Results

Microscopically: No cells were present in supernatant fluid of Specimen A, B, or C. In the residues of each, there were many packed leukocytes, predominantly granulocytes. In Specimen A, many of these cells were in the process of dissolution. In the residues of Specimens B and C, the cells were intact.

Lysozyme titre: In Specimen A, lysozyme was found in high titres in both the supernatant and cellular residue. In Specimens B and C, lysozyme was found in significant quantities only in the cellular residue. Repeating the experiment yielded essentially the same result.

Controls: Mucinase incubated with granulocytes had no appreciable effect. Mucinase did not depolymerize the substrate used in the lysozyme determination.

Thus to our satisfaction, the source of at least the major portion of the lysozyme found in the stools of one patient with ulcerative colitis was found to be the invading granulocyte which left lysozyme in active form after cellular breakdown.

We then turned our attention to the lysozyme titre of viable granulocytes found in the circulating blood of healthy adults. The method used to quantitate this titre follows. As a unit of measure, we chose the number of units of lysozyme found in 10^7 granulocytes.

TABLE I

Lysozyme and leukocytic content of cellular residue and supernatant fluid from isolated colonic loop in ulcerative colitis

	Specimen A (washings after 24 hours rest)	Specimen B (second washing 10 minutes after first irrigation)	Specimen C (third washing 10 minutes after second irrigation)
Supernatant	Micro: no cells Lysozyme: 16.9 units	Micro: no cells Lysozyme: 0 units	Micro: no cells Lysozyme: 0 units
Residue	Micro: many granulocytes; a large number in process of degeneration Lysozyme: 29.3 units	Micro: many granulocytes, mostly intact Lysozyme: 29.3 units	Micro: many granulocytes, all intact Lysozyme: 14.6 units

Method

A. Collection of blood

1. Prepare dry 50 ml. tube (plastic or glass) with a 20 ml. mark.
2. Add 0.4 ml. of 10% potassium oxalate solution (or other anti-coagulant).[2] Evaporate to dryness in 50 ml. tube to avoid volume error.
3. Prepare fresh fibrinogen solution (310 mg. fibrinogen in 10 ml. distilled H_2O or equivalent) (12).
4. Collect 20 ml. blood (from *dry* syringe or from blood bank), invert tube carefully several times to mix with anti-coagulant.

B. Separation of leukocytes

1. Add immediately 2½ ml. of the fibrinogen solution to 20 ml. of blood. Mix by inversion.
2. Allow to stand at a slight angle for about one hour or until the supernatant plasma is about half the total volume.
3. Carefully remove the supernatant plasma suspension with a capillary pipet and rubber bulb and transfer to a graduated centrifuge tube (15 ml.) or hematocrit tube.
4. Remove small sample of suspension, after mixing well, for WBC counts and differentials.
5. Centrifuge for about five minutes at full speed.
6. Note total volume of plasma and packed cells.
7. Remove clear supernatant plasma to the 1 ml. mark, thus leaving the packed cells in 1 ml. volume of plasma.

[2] Heparin has been shown to be a mild inhibitor of free lysozyme. However, it has little effect on lysozyme contained in granulocytes as determined by comparison with oxylated blood.

TABLE II

Lysozyme content of 10^7 granulocytes in healthy adults

Patient	WBC of plasma suspension	% of granulocytes	Total lysozyme (1 ml. of plasma suspension)	Plasma suspension (*ml.*)	Lysozyme per 10^7 granulocytes
J. D.	9,000	30	9.1	3.2	9.7
B. M.	8,900	53	13.3	9.5	11.9
?	9,800	65	25.3	4.2	9.5
R. H.	12,850	59	18.9	4.7	5.4
R. H.	6,520	64	20.1	6.0	8.0
R. H.	4,900	73	6.3	3.5	5.3
Duplicate	8,100	56	8.9	4.2	4.7
J. F.	4,720	38	9.9	6.0	9.2
Duplicate	11,520	66	37.9	6.0	8.3
A. B.	13,640	71	44.9	7.5	6.2
Duplicate	14,040	73	35.9	7.0	5.0
H. D.	7,200	11	4.6	5.3	11.5
C. F.	7,500	27	9.8	6.0	8.0
Average					8.0

Lysozyme content of 10^7 granulocytes in diseased states

Patient		WBC of plasma suspension	% of granulocytes	Total lysozyme of plasma	Plasma suspension (*ml.*)	Lysozyme per 10^7 granulocytes
O. F.	Ulcerative colitis	7,000	52	26.6	6.0	12.2
T. S.	Ulcerative colitis	4,206	31	4.4	6.0	5.6
L. P.	Cirrhosis of liver	9,500	61	36.7	9.0	7.0
A. M.	Rheumatoid arthritis	8,580	62	40.8	8.6	8.9
J. K.	Regional ileitis	10,100	80	23.4	8.4	3.5
J. K.	Chronic infection	15,000	76	77.6	9.4	7.3
J. M.	Lung abscess	8,280	36	22.2	9.7	7.7
W. W.	Chronic pancreatitis	11,960	65	29.8	7.4	5.2
Average						7.3

8. Three ml. of N/10 HCl are added to tube with 1 ml. of packed cells and plasma, stirred up and transferred quantitatively to tissue grinder (in ice bath). Repeat two more times. Therefore, a total of 9 ml. N/10 HCl is added, diluting the cell plasma suspension ten-fold.
9. Extract is ground ten minutes, transferred to centrifuge tube and centrifuged about seven to eight minutes.

Viscosimetric determination of lysozyme is done on this extract.

C. Calculation of lysozyme in granulocytes

1. Calculate units of lysozyme per ml. of plasma-cell suspension.
2. Calculate the total number of granulocytes in total volume of plasma cell suspension from WBC and differential.
3. Lysozyme per 10^7 cells equals lysozyme units/ml. of plasma-packed cell suspension (contains total number of granulocytes) divided by

$$\frac{\text{total number of granulocytes}}{10^7}$$

Accuracy: Because of the great number of possible sources of error, the utmost precautions were taken, particularly in the obtaining of the granulocyte count from the total leukocyte count and differential. In four duplicate experiments, the range of difference was 12% (Table II). In spite of this large factor of error, it is obvious that the lysozyme titre in the granulocyte of normal individuals has a great deal of variation, the lowest being 4.0 units per 10^7 granulocytes and the highest being 12 units per 10^7 granulocytes. There is also a fluctuation from day to day in one individual although only over a range of plus or minus 4 units.

In the disease states studied, the average unit of lysozyme per 10^7 granulocytes was approximately the same as in normal controls. This was true even of disease associated with a tremendous daily loss of granulocytes.

Sympathomimetic agents (adrenalin) and parasympathomimetic agents (mecholyl) did not alter the titre of lysozyme in the granulocytes of one normal adult. This was shown by giving a large dose of each drug, and 20 minutes later determining the lysozyme titre of the granulocytes.

Normal level—5.7 units per 10^7 granulocytes
After 20 mg. mecholyl I.M.—6.5 units per 10^7 granulocytes
After 1 ml. adrenalin (Parke-Davis)—6.2 units per 10^7 granulocytes

The adrenalin produced a marked leukocytosis without altering the lysozyme titre.

Another possibility requiring consideration was the capacity of granulocytes to pick up lysozyme from the surrounding media. In order to determine whether or not living granulocytes are able to pick up lysozyme *in vitro*, the following experiments were carried out:

1. Twenty to 25 ml. of blood from one normal donor were collected over heparin in each of two tubes and a plasma suspension of leukocytes separated in accordance with the Minor-Burnett procedure. Counts were made on the cell suspension.

2. To a measured volume of plasma suspension in the first tube, 2 ml. of 0.9% NaCl were added; to an equal volume of plasma suspension in the second tube, 2 ml. of egg-white lysozyme solution (prepared from Armour egg-white lysozyme), equivalent to a total of 200 micrograms or 210–270 units, were added.

3. The tubes were mixed by inversion and placed in a

water bath at 37°C for ½ to 3¾ hours, during which time they were mixed intermittently.

4. The suspensions were centrifuged and lysozyme determinations made on the packed cells and on the supernatant plasma.

In two separate experiments, the lysozyme added was almost completely recovered in the supernatant plasma, and the concentration of lysozyme in the packed cells of the control tube was essentially the same as that in the experimental tube. A third experiment was run, substituting a diluted solution of human tears for egg-white lysozyme solution, and similar results were obtained. It was apparent then, under the conditions of our experiments, that living leukocytes do not pick up lysozyme from the surrounding medium of plasma.

DISCUSSION

The high concentration of lysozyme which is present in the colonic exudate in patients with ulcerative colitis has been correlated in the present studies with the presence of large numbers of granulocytes. When steps were taken to separate the leukocytes from the mucus in such exudate, it was found that the mucus fraction was devoid of lysozyme activity unless considerable autolysis of the white cells had occurred. With degradation of the white cells, lysozyme in active form remained present in the surrounding medium. It seems clear, therefore, that the high concentration of lysozyme which is found in the stools of patients with ulcerative colitis is derived from granulocytes.

In the present investigation it has also been shown that substantial quantities of lysozyme are present in the circulating granulocytes of both normal and diseased individuals, in confirmation of the previous work of Gottschall and others. It appears, therefore, that the lysozyme in the feces in ulcerative colitis is derived primarily from the circulatory granulocyte.

Theoretically, it seemed possible that the granulocyte might absorb lysozyme from some external source. *In vitro* studies with suspensions of lysozyme, both egg-white and human, and granulocytes failed to yield any evidence that such absorption did occur. This possibility, therefore, appears unlikely.

It was of interest that the colonic exudate of patients with ulcerative colitis in symptomatic remission contained large quantities of leukocytes and large amounts of lysozyme. All of the patients who were subjected to this study continued to present gross evidence of inflammation of the rectum on proctoscopic examination. Further studies of this type on individuals in symptomatic remission, with proctoscopic findings which have reverted to a normal picture, seem indicated.

Our studies do not conclusively solve the question of a destructive action of lysozyme on colonic mucosa which might contribute to the perpetuation of inflammatory changes in the colon in ulcerative colitis. The possibility remains that this enzyme may contribute to the continuation of the inflammatory process. The weight of evidence, however, supports the view that the lysozyme in the bowel is only a manifestation of the extent of leukocytic infiltration.

SUMMARY

1. The primary source of the lysozyme found in the stools of patients with ulcerative colitis is the invading granulocyte.

2. The lysozyme remains in active form following dissolution of the white cell body.

3. The lysozyme titre of the lining mucus obtained from the rectum in patients with ulcerative colitis remains high in spite of complete symptomatic remission of the disease.

4. The average lysozyme content of the granulocyte is 8 units per 10^7 cells with considerable individual variation and slight daily variation in the same individual.

5. Administration of sympathomimetic (adrenalin) and parasympathomimetic (mecholyl) drugs did not alter the lysozyme content of the granulocyte.

6. The granulocyte has no capacity to pick up human or egg-white lysozyme from a surrounding medium of plasma.

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TWO CASES OF CALCINOSIS RENIS, STUDIED BY MEANS OF RENAL BIOPSY AND RENAL FUNCTION TESTS[1]

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Nephrocalcinosis may either be due to calcification of necrotic tubular epithelial cells and of precipitates such as occur in corrosive sublimate poisoning, in tubular nephritis (lower nephron nephrosis) (1) and rarely in chronic glomerulonephritis (1–3), or it may appear as part of a metastatic calcification due to mobilization of skeletal calcium in such conditions as hyperparathyroidism, hypervitaminosis D, osseous metastases (4), myelomatosis, osteomalacia, and long-term immobilization of bones (trauma, tuberculosis, and osteomyelitis). Finally, it may be seen following abnormalities of the blood chemistry (other than hypercalcemia) such as hyperchloremic acidosis and hypochloremic alkalosis.

A comprehensive review of nephrocalcinosis in human pathology has recently been given by Geraci, Harris, and Keith (5), and the literature concerning experimental metastatic calcification in the kidneys has been collected by Dick and Prior (6).

In the present paper two cases of calcinosis renis are reported. One patient suffered from a parathyroid adenoma with hyperparathyroidism, the other from calciferol poisoning. In both cases there was a secondary renal disease, and the renal biopsy method afforded essential information about its nature. Differentiated renal function studies were also made in these two patients; the results could be compared with the histological examination undertaken simultaneously.

Renal lesions in hyperparathyroidism

In a paper on renal complications in hyperparathyroidism based on 83 cases, Albright, Baird, Cope, and Bloomberg (7) have divided the renal changes in this disease into the following three groups: *1*) deposition of calcium in the renal pelvis with secondary pyelonephritis, *2*) deposition of calcium in the tubules of the kidney with ensuing sclerosis, renal contraction and insufficiency, and *3*) deposition of calcium in the kidney as well as in other organs, with a resulting acute renal insufficiency and death in the course of a few hours or days. These three types occurred in 27%, 23%, and 1%, respectively, of their series of hyperparathyroidism.

One of the cases reported here belongs to the second of these groups. This type of renal change will most frequently be localized chiefly in the medulla of the kidney. It consists of interstitial fibrosis, infiltration with lymphocytes and plasma cells, cystic tubular dilatation, thickening and, in some cases, calcification of tubular basement membranes, a relative absence of active glomerulitis or involvement of tubular epithelial cells and, as the fundamental lesion, interstitial deposition of calcium, according to Anderson (8), localized chiefly peritubularly. Bell (9), on the other hand, considers that the calcium, to some extent, is found within the tubules, partly because the calcium is found in cystic cavities which, he supposes, are dilated collecting tubules, and partly because it also is found in the form of deposits of the same shape and direction as collecting tubules (in which areas the tubular epithelium has either disappeared completely or has become calcified). Both authors agree that the impairment of the renal function is due to the obstruction of the tubules.

Hyperparathyroidism. Case report

In November, 1950, we had the opportunity of examining a case of hyperparathyroidism in a 30-year-old woman (E. N. R.).

The patient had been in good health until, in 1943, she became pregnant. In the course of pregnancy she developed a severe toxicosis which necessitated interruption of the pregnancy in the fifth to sixth month. On examinations made on this occasion, there was no proteinuria, and the specific gravity of the urine varied from 1,006 to 1,020. Since then the patient had been constantly ill,

[1] Aided by a grant from King Christian X's Foundation.

though her condition varied. The symptoms were pronounced fatigue and pain "all over the body." In addition, she had had periodical vomiting for months with great loss of weight, polydipsia and polyuria. In 1946 she had intense left-sided lumbar pain for a period of time. She had periodical headache and flickering before her eyes, and during recent years, dyspnoea on exertion. The patient had been in hospitals repeatedly, but the diagnosis was not established until the autumn of 1950.

On physical examination (November, 1950) the patient was pale, lean, and of a tired appearance. There were no palpable tumours of the neck. The thoracic column displayed a slight dextroconvex scoliosis. Intravenous pyelography showed somewhat coarse, but slightly dilated pyelograms and normal ureteral shadows. There were no visible calcifications in the soft parts, especially not in the kidneys, and no renal calculi.

On laboratory examination, the following was discovered: serum calcium 15.6 mg. %, serum phosphorus 1.1 mg. %, alkaline phosphatases 31.4 and 59.6 units (Buch's method; Normal Value 2.0–9.4 units). The urine contained traces of protein, the hemoglobin percentage was 78, blood pressure 125/80 mm. Hg, and serum creatinine 0.76 mg. %, NPN 24 mg. %, and blood urea 27 mg. %.

The results of the differentiated renal function test are shown in Table I. The glomerular filtration rate (expressed by the inulin clearance and the creatinine clearance) and the urea clearance, as well as the maximal tubular secretion (expressed by the para-aminohippuric acid-Tm [Tm_{PAH}]) and the renal plasma flow (expressed by the para-aminohippuric acid clearance [C_{PAH}]) were reduced to a little more than half the normal. The

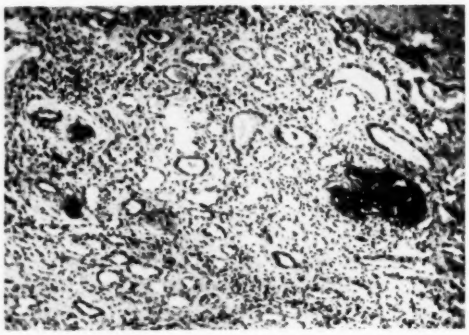

FIG. 1. SECTION OF KIDNEY BIOPSY FROM PATIENT WITH HYPERPARATHYROIDISM (CASE 1)

Medulla with calcium deposits, apparently both interstitially and intracanalicularly. Slight interstitial fibrosis and slight inflammatory cellular infiltration. (× 125)

filtration fraction showed a very slight increase, the ratio between inulin clearance and para-aminohippuric acid-Tm (C_{IN}/Tm_{PAH}) was normal and the ratio between para-aminohippuric acid clearance and the para-aminohippuric acid-Tm (C_{PAH}/Tm_{PAH}) was a little below the normal.

Biopsy of the kidney (according to the technique described by Iversen and Brun [10]) was made three days before the examination of the renal function. The biopsy material measured about 13 mm. in length and consisted of equal parts of cortex and medulla. Histological examination

TABLE I

Differentiated kidney function examinations of two patients suffering from calcinosis renis

The kidney function has been re-examined 12 and 18 months after the first examination. It has not been corrected for surface area.

No., initials, date	Diagnosis	Inulin clearance	Creatinine clearance	Urea clearance	PAH clearance	Tm_{PAH}	Blood urea	Serum creatinine	C_{IN} Tm_{PAH}	C_{IN} C_{PAH}	C_{PAH} Tm_{PAH}	Weight	Height
1. E. R. Nov., 27, 1950	Hyperparathyroidism	55.2	70.5	47.3	216	28.9	27	0.76	1.91	25.6%	7.5	48.4 Kg.	160 cm.
Dec. 5, 1951		42.8	58.7	25.2	251	—	52	1.32	—	17.1%	—	63.5 Kg.	—
2. G. Y. A. May 12, 1950	Dg-poisoning	42.9	53.3	26.4	308	30.3	42	1.5	1.42	13.9%	10.2	56.8 Kg.	170 cm.
Nov. 11, 1951		75.6	68.6	39.3	397	—	43	1.37	—	19.1%	—	65.0 Kg.	—

showed quite large calcium deposits both in the cortex and medulla, especially in the latter (Figures 1–3). It was difficult to decide whether they were interstitial or intracanalicular, but some of them, at any rate those in the medulla (Figure 1), were unquestionably intracanalicular. In a single section the cortex contained about 15 glomeruli, of which three seemed to be completely intact. A few of the others were partly hyalinized (Figure 2), the rest were completely hyalinized (Figure 3). In five different sections 64 glomeruli were found. Thirty of these were completely hyalinized, 17 slightly to moderately hyalinized, and 17 were normal. There was a considerable fibrosis around the hyalinized glomeruli, and the fibrous tissue here displayed a rather intense diffuse infiltration with lymphocytes and histiocytes and very few plasma cells (Figure 3). In addition there was a slight diffuse interstitial fibrosis in the cortex and quite a number of atrophic tubules around the perishing glomeruli. Otherwise the tubules did not seem to display anything abnormal. The lumina of both the proximal and distal convoluted tubules were rather wide; there were no casts. There was quite a pronounced increase of connective tissue in the medulla with rather intense diffuse lymphocyte and histiocyte infiltration. The few vascular lumina found in the biopsy material did not seem to display anything unquestionably abnormal. However, in a few small arteries there was possibly a slight hyaline thickening. Phosphatase staining (by the method of Gomori) was distinctly positive in almost all lumina of the proximal convoluted tubules that were intact. There was undoubtedly also a positive phosphatase staining in a few Henle's loops, of which the biopsy material, however, contained very few. It is possible that the increased amount of calcium in the tissue may have contributed to the positive phosphatase test, as the latter depends on a precipitation of calcium.

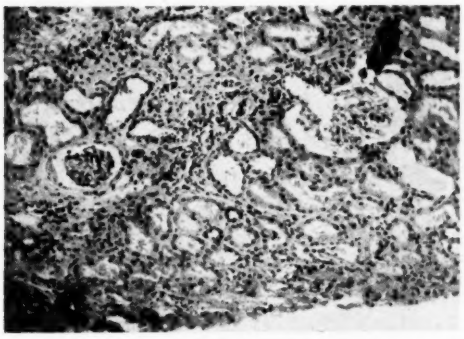

FIG. 2. SECTION OF KIDNEY BIOPSY FROM THE SAME PATIENT AS FIGURE 1

Cortex with a normal and a slight hyalinized glomerulus, interstitial calcium deposits, slight interstitial fibrosis, tubular atrophy and mild inflammatory cellular infiltration. (× 125)

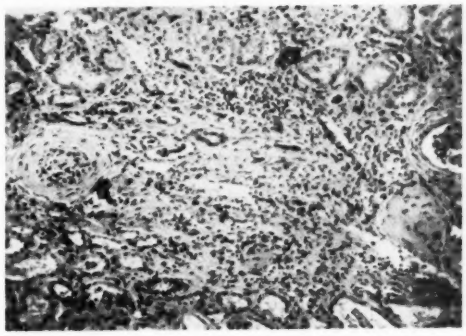

FIG. 3. SECTION OF KIDNEY BIOPSY FROM THE SAME PATIENT AS FIGURES 1 AND 2

Cortex with hyalinized glomeruli, interstitial calcium deposits, pronounced interstitial fibrosis, tubular atrophy and mild inflammatory cellular infiltration. (× 125)

Summary of the histological description. Quite considerable quantities of intracanalicular and interstitial calcium deposits were found both in the cortex and the medulla. Further, there were interstitial fibrosis and numerous hyaline glomeruli. In January, 1951, a parathyroid adenoma was successfully removed from the superior thoracic aperture. Microscopic diagnosis revealed eosinophilic adenoma of the parathyroid gland.

Re-examination of the kidney function about one year later (see Table I) showed that the glomerular filtration (inulin and creatinine clearance) had been reduced to 75–80% of what it had been in November, 1950. The blood urea and serum creatinine were increased to pathological values (52 and 1.32 mg. %), while the para-aminohippuric acid clearance was unchanged.

Renal lesions in calciferol poisoning

It has been demonstrated by numerous authors (11), in various experimental animals, that cal-

cium deposits and calculus formation occur in the kidneys in hypervitaminosis-D. It has been shown by Guldager (12) that the calcium deposits were found in particular in the convoluted tubules and in the small vessels and capillaries of the cortex, whereas the glomeruli remained unaffected. Goormagtigh and Handovsky (13) have found hypertrophy of the tunica media of the arterioles and injury of the tubules which might lead to uremia.

In human beings, too, a prolonged treatment with vitamin D_2 may cause a progressive renal disorder with proteinuria, hematuria, casts in the urine, and a decrease of the renal function, possibly with hypertension (11). Corcoran, Taylor, and Page (14) have made a differentiated renal function test in two patients with reduced kidney function from intoxication with irradiated ergosterol. In the cases examined, the histological changes in the kidney occurred in the form of calcium deposits in the medulla, chiefly localized in the lumina of the collecting tubules. Mention is also made, by Bauer and Freyberg (15), of calcium deposits in the vessels and of "arteriosclerotic nephropathy."

Calciferol poisoning. Case report

In April, 1950, a 28-year-old woman was admitted to the Medical Department III of Kommunehospital, for nephritis and observation for uremia.

In 1936 the patient had been treated for non-toxic struma with subtotal strumectomy. As the struma recurred with compression of the trachea, another strumectomy was made in 1940; it was extremely difficult because of severe adhesions. After the operation the patient was given calcium and A.T. 10 because of parathyroprival tetany.

In 1942 the patient had been given Ultranol ultraconcentrate, 50,000 i.u. and four calcium tablets (2 g.) per day. In the beginning her serum calcium was controlled every month, later every six months; during the last two years it had not been controlled at all. During the same period her ultranol intake had varied.

In April, 1949, the patient was feeling tired and poorly. Her doctor found that the sedimentation rate had increased (58 mm.), the hemoglobin percentage had fallen (66%), and further ascertained the presence of proteinuria of slight degree, normal blood pressure, and slightly increased blood urea (48 mg. %). Her condition has fluctuated considerably since then. In January, 1950, she felt ill again with nausea, vomiting, anorexia, and fatigue. On examination her doctor found the following: slight anemia (72%), increased sedimentation rate (180 mm.), increased blood urea (54 mg. %), greatly increased serum calcium (18.4 mg. %), and slightly increased serum phosphorus (5.1 mg. %).

The physical examination showed that the patient was pale and lean, but otherwise nothing abnormal was found. Intravenous pyelography showed a rapid and intense excretion in normal pyelograms in both kidneys. No calcification of the renal parenchyma and no renal calculi were observed. Roentgen examination of the femur and the humerus showed normal osseous structure without any signs of decalcification.

Laboratory examinations in April, 1950, revealed the following: blood pressure 130/80–110/70 mm. Hg, hemoglobin percentage 67, blood urea 42 mg. %, serum creatinine 1.5 mg. %, alkaline phosphatases 4.3 units (Buch), serum calcium 11.8 mg. %, serum CO_2 35.6 mmol., serum chloride 98.0 meq./L. The urine contained no protein on repeated examination, and several microscopic examinations showed it to be normal.

The differentiated renal function tests showed that the inulin clearance, the para-aminohippuric acid clearance and the para-aminohippuric acid-Tm were reduced to about half the normal. The inulin clearance/para-aminohippuric acid-Tm (C_{IN}/Tm_{PAH}) and the filtration fraction showed low normal or slightly reduced values, whereas the ratio between the para-aminohippuric acid clearance and the para-aminohippuric acid-Tm (C_{PAH}/Tm_{PAH}) was normal.

The concentration capacity of the kidneys (determined according to Addis-Shevky's method) was impaired (specific gravity: 1,014).

Biopsy of the kidney was made twice with an interval of three weeks (Figures 4–5). At the first biopsy, a specimen measuring about 6 mm. in

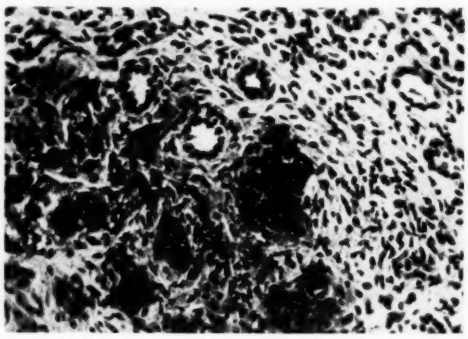

FIG. 4. SECTION OF KIDNEY BIOPSY FROM PATIENT WITH D_2 POISONING (CASE 2)

Medulla with calcium deposits, apparently mainly intracanalicularly. Slight interstitial fibrosis and mild inflammatory cellular infiltration. (× 140)

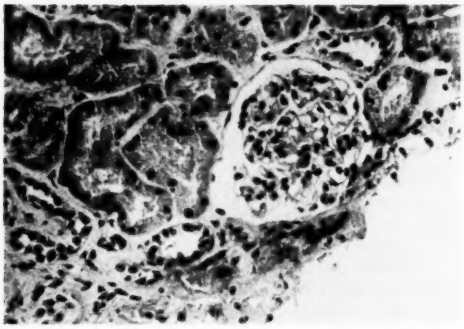

FIG. 5. SECTION OF KIDNEY BIOPSY FROM THE SAME PATIENT AS FIGURE 4

Normal cortex. (× 280)

length was removed. It consisted chiefly of medulla. This contained a number of large calcium deposits, localized, as far as could be determined, mainly within the canaliculi (Figure 4). There was a slight increase of connective tissue and a slight diffuse interstitial lymphocyte and histiocyte infiltration; otherwise nothing abnormal was found.

At the second biopsy, three weeks later, a piece of cortex, measuring about 6 mm. in length was removed. It contained 12–14 glomeruli and displayed no definite abnormalities. There were no calcium deposits and the glomeruli were normal (Figure 5). In a "differential count" in four different sections of about 50 glomeruli, a single completely hyalinized glomerulus was found, whereas all other glomeruli were normal. There were tall, granular cells of normal appearance in the proximal convoluted tubules. At the border of the medulla, there was a slight increase of connective tissue, a slight diffuse infiltration with lymphocytes and histiocytes, and many cellular remnants. Phosphatase staining (Gomori's method) showed a faintly positive reaction in parts of the proximal convoluted tubules.

During the patient's stay in the hospital, the renal function improved considerably, as the 24-hour creatinine clearance rose from 34 to 59 ml./min. in the course of three weeks.

The kidney function was re-examined 18 months later (see Table I). The glomerular filtration (inulin, creatinine, and urea clearance) had increased considerably. The para-aminohippuric acid clearance had also increased, while the blood urea remained unchanged and the serum creatinine had decreased.

DISCUSSION

In both these cases of calcinosis renis, the histological findings in biopsy of the kidney correspond exactly to previously published findings in autopsy of patients who had died of hyperparathyroidism and D_2 poisoning. As far as we know, differentiated renal function tests have been reported previously only in cases of D_2 poisoning (13), not in cases of hyperparathyroidism. In both patients in this study, about 50% reduction of both the glomerular filtration and maximal tubular secretion was found. It was possible in both cases to compare the function and the amount of pathological and normal glomeruli. In the case of hyperparathyroidism, about half the 64 glomeruli examined had presumably been completely destroyed. On the assumption that the renal tissue examined is representative of the histology of the entire kidney, it must be said that in this case, there was quite good conformity between glomerular filtration rate and the number of uninjured glomeruli (even if some of these also displayed slight pathological changes).

On the other hand, in the case of D_2 poisoning, only one single hyalinized glomerulus was found, whereas all other glomeruli were normal and, in spite of this, the same reduction in glomerular filtration rate was found as in the case of hyperparathyroidism.

It cannot be settled on the basis of the present conditions, whether this discrepancy between decreased kidney function and normal histological findings is due to functional alterations, which cannot be observed in the histological preparations, or whether it is due to the fact that the biopsy was not representative of the histological changes in the kidneys. From the literature it seems reasonable to suppose that D_2 poisoning will cause diffuse kidney injuries. The fact that the kidney function in this case has improved considerably might also indicate that the injuries were functional, or possibly due to a reversible and not too extensive nephrocalcinosis. The various degrees of glo-

merular destruction which have been found in these two patients, in spite of the identical reduction in their kidney function, correspond very well to the changes which have later been found in their kidney function, after treatment of the underlying diseases. The kidney function of the first patient, whose glomeruli were mainly hyalinized, has decreased. In the other patient, whose glomeruli were histologically normal, the kidney function has increased.

SUMMARY

A case of hyperparathyroidism in a 30-year-old woman, and one of vitamin D_2 poisoning in a 28-year-old woman are reported. In the first patient, biopsy of the kidney showed considerable calcium deposits in the cortex and the medulla, interstitial fibrosis and numerous hyaline glomeruli. In the second patient, biopsy of the kidney showed calcium deposits in the medulla only, localized in the collecting tubules, and normal glomeruli. In renal function tests, the glomerular filtration rate and the maximal tubular secretion were reduced by about 50% in both patients. At the re-examination, one and 1½ years later, respectively, the kidney function of the first patient had decreased, while in the second patient, it had increased. This finding might indicate the prognostic value of the kidney biopsy.

ACKNOWLEDGMENT

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STUDIES ON ADRENOCORTICAL EOSINOPENIA: A CLINICAL AND STATISTICAL EVALUATION OF FOUR-HOUR EOSINOPHIL RESPONSE TESTS [1, 2]

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As early as 1914 Schwarz (1) suspected an inverse relationship between adrenal activity and the level of circulating eosinophils. Twenty-five years later Dalton and Selye (2) noted eosinopenia following stress in animals and related it to release of adrenal cortical hormones. More recently Thorn and his associates (3, 4) observed a prompt decrease in circulating eosinophils in healthy persons, and in patients, following a single injection of pituitary adrenocorticotropic hormone (A.C.-T.H. or corticotropin). The greatest reduction in eosinophils was seen four hours after injection and was related to secretory activity of the adrenal cortex. In a study of 40 people with normal adrenal activity Thorn, Forsham, Prunty, and Hills (5) reported that the mean drop in circulating eosinophils at four hours was 70% below pre-injection levels. In all instances the drop in circulating eosinophils was greater than 45%. This was in sharp contrast to what was seen in patients with Addison's disease, where they noted little change in circulating eosinophils after injection of corticotropin. Thorn, therefore, proposed that the eosinophil response to A.C.T.H. should be used as a test in the diagnosis of Addison's disease. He and Forsham suggested (6) that a four-hour post-injection ". . . fall of 50% or more of the circulating eosinophils eliminates the diagnosis of adrenal cortical insufficiency," and they indicated that an inadequate response, of less than 50% fall, may represent true adrenal cortical insufficiency.

A transitory eosinopenia also develops in animals after injection of epinephrine. On the basis of extirpation and electrical stimulation studies on dogs by Hume and Wittenstein (7, 8), Recant, Hume, Forsham, and Thorn (9) postulate that the hypothalamus, pituitary, and adrenal cortex are successively stimulated to produce epinephrine eosinopenia. They advocate a four-hour eosinophil response test to parenteral epinephrine for estimating the functional activity of the pituitary-adrenal axis in man.

In this laboratory, working with Samter, we found that oral ephedrine is also capable of producing transient eosinopenia in man (10–13). This phenomenon has been observed independently by Abelson and Moyes (14). While the four-hour response of eosinophils to epinephrine or ephedrine has been used mainly in testing individual patients, it has also been used to measure pituitary-adrenal activity in various physiological states, such as infancy (15), old age (16), following operations (17), and to assess groups of people ill with different types of disease processes such as chronic alcoholism (18) and acute gout (19).

In order to test the validity of the different eosinophil response tests as clinical tools in diagnosis and as indices of activity of the hypothalamus, pituitary, or adrenal cortex, observations were made on a large number of patients; among whom were many with conditions thought to be related to abnormalities of the pituitary-adrenal axis. The data obtained were subjected to statistical analysis and are reported below.

METHODS

Selection of patients. Patients were selected who had Addison's disease, known pituitary tumors, suspected hypopituitarism, prolonged inanition, cachexia, or unusual eosinophilia. Others were chosen at random from hospital admissions over a two-year period, and a few healthy persons were included in the study. In all, 284 individuals were studied. Their ages ranged from 12

[1] Supported in part by a grant from Armour and Company, Chicago, Ill.

[2] Presented at the 24th Annual Meeting of the Central Society for Clinical Research, November 2 and 3, 1951, Chicago, Ill.

[3] Armour Research Fellow in Medicine.

to 72 years. Eleven % were in their teens and the remainder were rather evenly distributed between 20 and 70 years, the mean age being 40 years. Fifty-seven % were female. The most frequent diagnoses included cardiovascular disease (15%), diabetes mellitus (9%), rheumatoid arthritis (7%), neoplasms and leukemia (7%), bronchial asthma (6%), chronic and subsiding infections, miscellaneous pulmonary conditions, and psychoneurosis (4% each). There were nine patients who were thought to have Addison's disease, but only two were demonstrated to have true adrenocortical insufficiency. Five patients had tumors in, or encroaching upon, the pituitary body. Hypopituitarism was suspected in seven patients ill with marked weight loss but confirmed in none.

Technique of testing. Patients believed to be in acute stress due to their illness or its treatment were not tested. Patients with significant hypertension, coronary artery disease, or arteriosclerosis were not tested with epinephrine or ephedrine.

Tests were scheduled so as not to coincide with other major diagnostic or therapeutic procedures. Regular ward activities were not restricted. When feasible, daily medications were withheld until completion of the test. In all patients fasting venous blood was drawn at 8:00 a.m. and placed in a tube with heparin.

Following this the patient was given tablets of ephedrine sulfate, 45 mg. orally, or corticotropin,[4] 20–25 mg. intramuscularly or green placebo capsules, orally. Epinephrine, when given, was administered in amounts of 0.2 to 0.4 mg. intravenously over a one-hour period, or 0.3 mg. subcutaneously. A light breakfast was allowed, but lunch was withheld until the four-hour blood specimen had been drawn at noon. For each test the same individual made both eosinophil counts. Results of the test were recorded as per cent decrease in eosinophils after four hours, below the fasting level.

Repeat tests on a patient were usually scheduled two or more days apart. The sequence with repeated tests was not always the same, but generally two or more tests with ephedrine sulfate or epinephrine would be followed by placebo tests, and finally corticotropin tests would be made. In all, 702 four-hour response tests were carried out.

Technique of cosinophil counts. Chamber eosinophil counts were made using the phloxine-propylene glycol stain of Randolph (20) or as modified by Henneman, Wexler, and Westenhaver (21). Four standard 0.9 mm.[3] Levy chambers (two hemocytometers) were filled from a single pipette after a 1:10 dilution. The total number of cells counted in the entire ruled area of the four chambers was multiplied by 2.78 to give the number of eosinophils per mm.[3] of blood.

[4] Corticotropin was obtained through the courtesy of: Armour and Company, Chicago (25 mg. dose) and Wilson and Company, Chicago (20 mg. dose). A small quantity of corticotropin from an experimental batch made by another pharmaceutical company failed to produce eosinopenia. Studies with this preparation were not included in statistical analyses.

Statistical analysis. Standard techniques of quantitating variation and significance of group differences as outlined in Snedecor (22) were employed. Fiducial limits of the mean signify the 95% confidence limits.

RESULTS

Intrinsic error of single eosinophil counts

The intrinsic error of enumeration and physiologic changes in eosinophil level have been outlined previously by one of us with Samter (11), and have been further emphasized by Fisher and Fisher (23). Unfortunately, these sources of error have not been appreciated by all investigators utilizing this technique.

From the point of view of the present study, it was necessary to investigate the intrinsic error of chamber eosinophil counts in more detail than had been done previously so that valid statistical conclusions could be made on eosinophil counts obtained in the tests. Therefore, the standard deviation of a group of four individual chamber eosinophil counts was calculated for each of 500 blood specimens from this series. The first 400 were taken consecutively; the last 100 included only counts greater than 275 cells per mm.[3] as there were already sufficient observations regarding the lower mean counts. These standard deviations are plotted against the respective means of the four chamber counts as illustrated in Figure 1.

These values are compared with the curve of maximum possible standard deviation. This curve was plotted by inspection of all possible combinations of counts in four chambers which can yield representative mean eosinophil counts. The fewer the number of chambers in such a computation, the more markedly will this limiting factor restrict the correspondence of observed values as compared to the theoretical dispersion if an unlimited number of chambers were used. Inspection of this curve shows that maximal standard deviations were reached only when the mean eosinophil counts ranged from zero to 15 cells per mm.[3].

The observed standard deviations were also compared with a theoretical curve for one standard deviation of counts which was derived from the formulae of Berkson, Magath, and Hurn (24). By inspection of the data it can be seen that the observations are not equally distributed above and below the theoretical curve, the observed standard deviations being generally less than predicted,

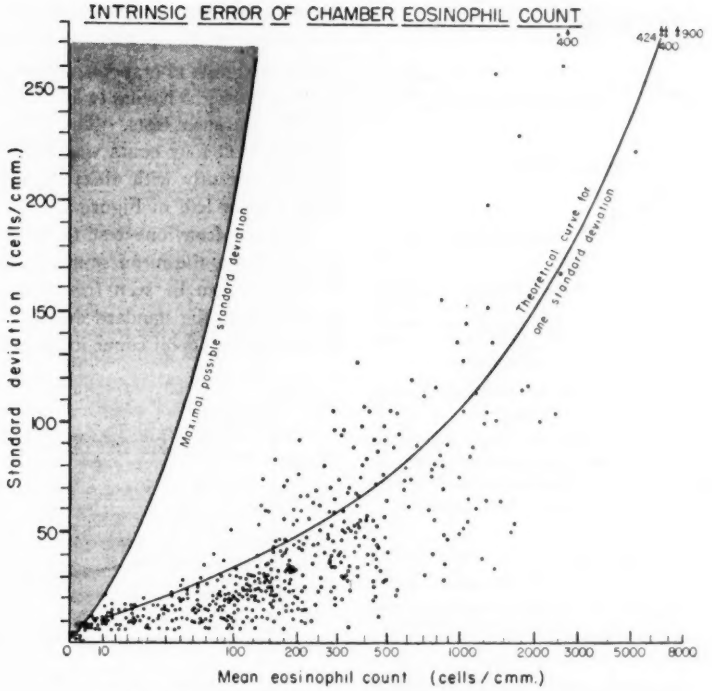

FIG. 1. INSTRINSIC ERROR OF CHAMBER EOSINOPHIL COUNTS

Each dot represents a series of four eosinophil counts from separate 0.9 mm.^{3} chambers filled by a single 1:10 blood-stain dilution. The calculated standard deviation for each such series is plotted against the mean eosinophil count of that series. Five hundred blood samples are thus represented. The theoretical curve for one standard deviation at any eosinophil level is derived mathematically from the formula of Berkson, Magath, and Hurn (24). The curve of "maximal possible deviation" was obtained from inspection of all possible combinations of counts from four chambers which can yield selected mean eosinophil counts. As only a few observations coincide with this curve, it does not appear to be an important limiting factor in the distribution of these data. The few dots appearing to exceed the maximal possible deviation actually coincide with it. Limited space on the graph necessitates this apparent discrepancy.

though having a parallel distribution. In spite of these apparent discrepancies, it seems advisable to use the theoretically derived values in assessing the probable intrinsic errors of the eosinophil count.[5]

TABLE I

Intrinsic errors of chamber eosinophil counts using one pipette with 1:10 dilution and four 0.9 mm.^{3} chambers

True eosinophil level of sample	Range for 95% of chamber counts on sample	Coefficient of variation
25 cells/mm.^{3}	8–42	34%
50 cells/mm.^{3}	26–74	24%
100 cells/mm.^{3}	65–135	18%
200 cells/mm.^{3}	148–252	13%
400 cells/mm.^{3}	323–477	10%

[5] Berkson, Magath, and Hurn (24) have pointed out the unconscious tendency towards faulty chamber counting by technicians and physicians. In their own studies they had to photograph each ruled area of the counting chamber and had to punch a hole through the photographic image of each cell to ensure *absolute* accuracy in cell counts made for statistical analyses.

In addition to the universal difficulty of counting objects as seen through the microscope, there are five situations encountered in the eosinophil counting chamber where "judgment" may be substituted for complete ob-

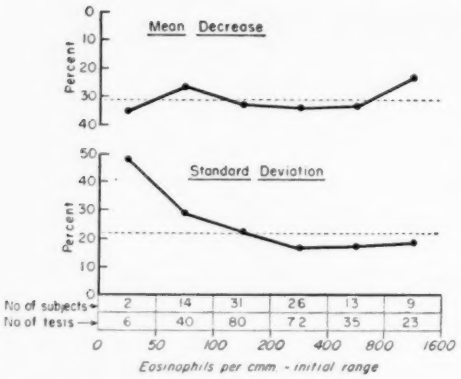

FIG. 2. TREND AND VARIABILITY OF REPEATED EPHEDRINE RESPONSE TESTS

Mean per cent decrease (above) and standard deviation (below) of repeated eosinophil response tests to 45 mg. ephedrine for each subject have been averaged and plotted against initial eosinophil levels (abscissa).

Table I presents the theoretical coefficient of variation and the two standard deviation limits (95% of observations) for several representative true eosinophil levels when all four chambers are used to compute the count, as was the case for all response tests reported herein. The coefficient of variation is greatest with low eosinophil levels despite the smaller range of cell counts.

jectivity. These are: (*1*) differentiation of eosinophils from debris, (*2*) differentiation of eosinophils from neutrophils if insufficient time is allowed for staining, (*3*) differentiation of eosinophils from eosinophilic staining erythrocytes which have been altered by incomplete removal of acetone from pipettes, (*4*) philosophy of enumeration when some eosinophils are clumped, and (*5*) determination of whether a given cell is inside or outside of the ruled area. Needless to say, one should examine the preparation under a higher power when doubt exists about the first three possibilities, and a new preparation should be made if the third or fourth exists. The fifth is relatively uncommon. Inasmuch as the total number of cells counted is usually small, even with four chambers, a difference of a few cells in a preparation can make an appreciable difference in comparison of mean counts and an even greater difference in calculation of the standard deviation. Thus, in our studies, if "judgment" were exercised in selecting or rejecting only two eosinophils per preparation of four chambers, and if this "judgment" were conditioned, as it usually is from training, by the desire to have consistent counts it would make a difference of approximately two cells per mm.^{3} in the standard deviation and would bring the observed values into line with the theoretical curve.

Variable response to ephedrine tests in the same subject

Repeated tests to oral ephedrine were performed in 95 subjects; 55 having two; 17, three; 21, four; and 3, five such tests. The mean decrease of eosinophils at four hours was 30.7% and did not vary significantly with different initial eosinophil levels (upper half of Figure 2).

Variation from one test to the next was frequently great, the mean standard deviation being 22.1%. It can be seen from the lower half of Figure 2 that the standard deviation decreased as the initial eosinophil count increased. Variability

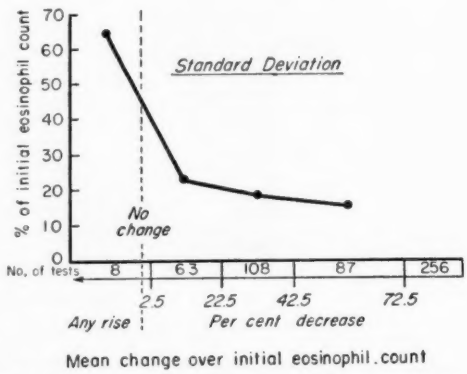

FIG. 3. VARIABILITY OF REPEATED EPHEDRINE TESTS ACCORDING TO MEAN DEGREE OF RESPONSE

Standard deviation of repeated eosinophil response tests (ordinate) to 45 mg. ephedrine for each subject have been averaged for each group and plotted against the mean percentage change in eosinophils after testing (abscissa).

of repeated tests was particularly marked with starting counts below 100 cells per mm.^{3}. With higher initial counts the standard deviation remained at about 18%.

The standard deviation of repeated tests in these same subjects was also studied with relation to grouped mean levels of response (Figure 3). This figure shows that the greater the tendency towards eosinopenic response to this test, the more consistent are the repeated tests.

Figure 4 shows that there was 2.4% greater mean response of all second over first tests. When they were repeated at very short intervals, second tests showed significantly less response than first

tests, but if three or more days elapsed between procedures, there was more than a 10% increase.

Although insufficient data of this type were collected for similar statistical analysis of repeated tests of subjects to epinephrine, corticotropin, and placebos, we have noted that these tests are also not accurately reproducible, and their degree of variability appears to be correlated with the same factors.

Comparative response to corticotropin, ephedrine, and placebos

The mean response to a given stimulus by each subject was analyzed with that of all other subjects so tested (excluding tests on patients with Addison's disease). Figure 5 shows the mean, the fiducial limits of the mean, the standard deviation, and the 95% confidence limits for all four-hour observations of the percent change in eosinophils from pre-treatment levels. These are grouped for tests with placebo, with ephedrine, and with corticotropin. The mean eosinophil decrease with placebo was 10.5%; with ephedrine, 40.8%; and with corticotropin, 62.0%. Values encompassed by fiducial limits of the respective means are widely separated.

It should be pointed out that in individual instances this difference may not be evident inas-

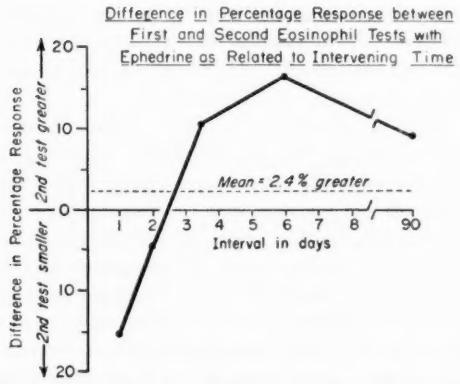

FIG. 4. DIFFERENCE IN RESPONSE BETWEEN FIRST AND SECOND EPHEDRINE TESTS

Percentage difference between first and second eosinophil response tests to 45 mg. ephedrine are related to the number of intervening days. The interval in 21 subjects was one day; in 19, two days, in 18, three or four days; in 18, five to seven days; and in 13, eight to ninety days.

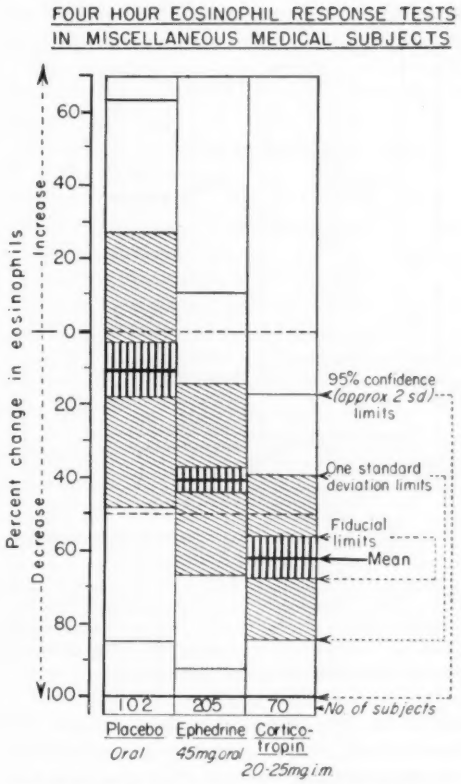

FIG. 5. EOSINOPHIL RESPONSE TESTS TO THREE AGENTS

Mean, fiducial limits of the mean, and one and two standard deviation limits are indicated for four-hour eosinophil response tests to placebo capsules, 45 mg. ephedrine, and 20–25 mg. corticotropin in a variety of patients.

much as the one standard deviation limits of all three groups overlap considerably. These limits encompass approximately two-thirds of observations for each group. Almost one-sixth of subjects given placebos showed four-hour eosinopenia of 50% or greater and in approximately one-sixth of non-Addisonian individuals the eosinophil levels fell *less* than 40% four hours after injection of corticotropin.

The mean levels of the three groups of tests and the standard deviations for each stimulus were analyzed in relation to the initial eosinophil level. The degree of response to placebo and cortico-

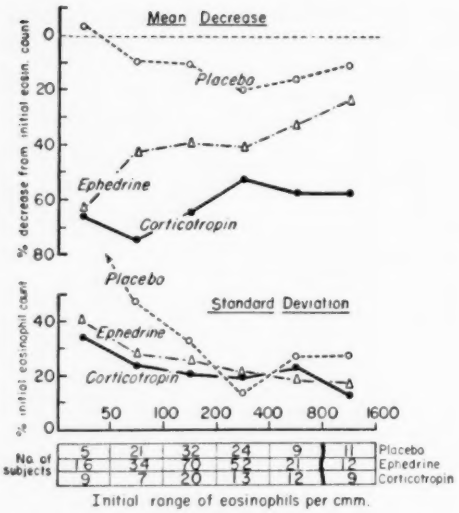

FIG. 6. TREND AND DISTRIBUTION OF RESPONSE TO THREE AGENTS

Mean per cent decrease (above) and standard deviation (below) of eosinophils in all subjects after four-hour response tests to placebo capsules, 45 mg. ephedrine, and 20–25 mg. corticotropin are grouped according to level of pre-test circulating eosinophils (abscissa).

tropin was little influenced by the mean eosinophil level, though there was a suggestion of decreasing response with high levels in tests using ephedrine (Figure 6). In all instances the standard deviations for each stimulus were greater with low than with higher counts.

Examples of variable responses in individual patients

Examples of the variability of the responses to the different tests are given below: The eosinophil level of a patient with refractory anemia fell 69%, 51%, 56%, and 35% and increased 7% after repeated tests to placebos; they fell 45%, 49%, and 34% after ephedrine testing; and they dropped 53% after injection of corticotropin. The eosinophil level of a young diabetic patient fell only 22% after corticotropin injection, but after ephedrine the eosinophil levels fell by 76%, 50%, 53%, and 64% on different occasions. Two patients with hypothyroidism were tested before and during thyroid therapy. The first of these had a 26% and a 29% fall with ephedrine before therapy, a 50% decrease the second week, and a 26% drop the third week of thyroid therapy. The second patient had a 21% decrease and a 10% rise before therapy, a 52% fall one week after thyroid extract had been started, and 26% and 11% decrease two weeks later.

Numerous other examples of wide variation in response to repeated tests with the same agent and seemingly paradoxical responses in comparison with tests to more than one agent could be cited.

Failure of response to corticotropin

Twenty-three subjects of the 76 tested with corticotropin at first failed to show a decrease of eosinophils exceeding 50%. Two of these had proven Addison's disease. In the first patient the eosinophils dropped 5%, 2%, and 23% in three separate tests with corticotropin and increased 3% and 8% after ephedrine. In the second patient there was a 4% and an 11% rise after corticotropin, a 12% fall after ephedrine and an 8% drop after injection of epinephrine.

One patient, ill with dermatomyositis had been tested during the time he was receiving cortisone and presumably he did not respond because of a temporary adrenocortical insufficiency. Five patients were tested with what turned out to be an inferior batch of corticotropin. Data from these six patients were not included in the statistical analyses. It is possible that some of the other failures were partially due to preparations of substandard potency as discussed later.

The subjects in whom initial response to corticotropin was "inadequate" had a greater than 50% eosinopenia after repeat testing with corticotropin and/or ephedrine. Unfortunately, circumstances beyond our control interfered with retesting five such patients. There was no clinical evidence, nor biochemical data to suggest adrenocortical insufficiency in any of these subjects, and it seems likely that a greater than 50% eosinopenia would have been noted in each of them with retesting.

Response to ephedrine in pituitary disease

It is well known that up to three quarters of the pituitary gland may be destroyed with few or no symptoms of hypopituitarism (25).

In the present study five patients with tumors arising within or infringing upon the sella turcica were given eosinophil response tests to ephedrine. Quantitative histologic data concerning viable pituitary tissue are not available on these cases. Responses varied from a 24% increase to a 65% decrease of eosinophils, with a mean fall of 38%.

Response to ephedrine in conditions of marked weight loss

Fifteen tests were performed on seven patients showing marked weight loss due to anorexia nervosa. Results ranged from a 9% to an 84% decrease after ephedrine with a mean drop of 51%. Six tests were done on four patients with carcinomatosis and cachexia. In them there was an 8% to a 100% fall in eosinophils and a mean drop of 41%. There was, therefore, no significant difference between the subjects of this group and those with other chronic illnesses insofar as their response to ephedrine is concerned.

DISCUSSION

Technical errors in eosinophil response tests

These studies amply demonstrate the lack of precision inherent in the four-hour eosinophil response tests. The errors of single eosinophil counts are basic to a consideration of variation in such tests. Some of the human errors of enumeration have been mentioned in footnote number 5 of this paper. In addition there is a chance variation related to the total number of cells counted. This theoretical error has been discussed previously (11) and is represented in Figure 1 and Table I for a single pipette and both sides of two standard hemocytometers. Exact accuracy of cell counts is hard to obtain with the best will in the world, but the common error of counting insufficient cells (using only one or two sides of a single hemocytometer) is easily avoided. We recommend at least four standard chambers (two hemocytometers), or preferably, four large Fuchs-Rosenthal chambers.

It is seen from Table I that the theoretical coefficient of variation for single specimens is greatest with low eosinophil counts. The error of comparing successive counts would be expected to reflect a similar relationship to initial eosinophil level. This was borne out in the standard deviations of Figure 2 for repeated tests in the same subject and in Figure 6 for the comparison of mean responses in many subjects. Thus, the greatest variation is noted with the lowest initial counts.

With higher initial eosinophil counts the intrinsic errors of counting became of less importance. In spite of this, a standard deviation of approximately 18% persists for repeated tests on subjects with high initial levels (Figure 2). This variability is apparently related to short term fluctuations in eosinophil level as described by Rud (26) as well as to alterations in the physiological state of the subjects. In order to minimize these factors it is important to see that the subject is under the least possible stress during the test.

As shown in Figure 3 the standard deviation of repeated tests was greater in subjects showing slight or no mean response than in those with a marked fall in eosinophils. This may be at least partially explained by the simple arithmetic fact that to yield mean values approaching a 100% decrease of eosinophils, values for repeated tests must be more closely grouped than is necessary for cases of lesser response.

The tendency towards greater response of second over first tests, particularly when separated by more than three days, as shown in Figure 4, may seem surprising. We feel this tendency is not significant inasmuch as early in the study repeat tests were only done on those subjects failing to show significant eosinopenia. In these patients, initial values were often lower than their mean response to subsequent repeated tests. More significant is the comparison of successive tests as related to the interval between tests. It appears that the responsiveness to ephedrine is impaired for several days after each test. We do not know whether this represents tachyphylaxis, fatigue, or adaptation. In any case, when tests are repeated they should be run at least three or more days apart.

Response to corticotropin in Addison's disease

The four-hour eosinophil response to a single injection of 25 mg. corticotropin, though a valuable tool in the diagnosis of Addison's disease, is certainly not infallible. We know of no instance in which a patient with Addison's disease has developed a significant eosinopenia to this test, but

we and others (27) have observed several patients without Addison's disease who failed to respond to injection of corticotropin. Therefore, failure of response to a *single* test with corticotropin cannot be considered diagnostic of Addison's disease.

It must be pointed out that in the past some experimental and commercial preparations of corticotropin were poor stimulators of the adrenal gland. Most of the corticotropin used in this study was tested for potency in healthy individuals. Nevertheless variations of potency may have been responsible for a few of the subnormal responses observed after injection of corticotropin.

In the event of an inadequate response repeated tests with corticotropin are indicated. The intravenous test (28), which gives a more powerful stimulus to the adrenal cortex than the intramuscular test, should be used in re-testing.

Epinephrine, ephedrine, and the hypothalamic-pituitary-adrenal axis

In the beginning of our study we used epinephrine as a stimulus for producing transitory eosinopenia. After performing over 50 tests with this agent (11) we changed to oral ephedrine which has very similar pharmacologic and physiologic effects on man; the main difference, according to Gaddum and Kwiatkowski (29), is that ephedrine is tachyphylactic and is less readily destroyed in the body. Within the broad limits of variability for the test, ephedrine is seen to produce approximately the same degree of eosinopenia in individual subjects as do the conventionally employed doses of epinephrine.

The clinical significance of the eosinophil response tests to epinephrine and ephedrine depend on whether or not these agents induce eosinopenia through successive stimulation of the hypothalamus, pituitary, and adrenal cortex in man. As far as we have been able to ascertain, Hume's work regarding the epinephrine effect on the hypothalamus of animals has not been repeated for either epinephrine or ephedrine (30). In the rat the hypothalamus is not a necessary part of the axis for the production of epinephrine eosinopenia. McDermott, Fry, Brobeck, and Long (31) demonstrated that application of minute amounts of epinephrine directly to a pituitary gland transplanted into the anterior chamber of the rat's eye would induce eosinopenia, whereas parenteral injection of the same quantity was without effect.

Sayers (32) has recently suggested that ". . . epinephrine does not discharge A.C.T.H. by a direct action on the adenohypophysis or via a neural link to the gland, but rather acts to increase peripheral utilization of cortical hormones." In this regard, Robinson (33) has studied a patient with rheumatoid arthritis, made unresponsive to corticotropin by cortisone. When epinephrine was given to him there was an appreciable drop in eosinophil count. Three of our patients with Addison's disease did not show significant eosinophil responses to epinephrine while on cortisone and two responded (three of these cases were recently added to the series, and were not considered in previous discussions).

However, we have studied five patients with carcinoma of the prostate after complete adrenalectomy while they were receiving cortisone (34). Following 0.3 mg. epinephrine subcutaneously, eosinophils decreased by 36 to 78% with a mean fall of 61%. Little change in level of circulating eosinophils followed injection of saline or corticotropin.

As long as the mechanisms of epinephrine and ephedrine induced eosinopenia are not fully clarified, the interpretation of inadequate response to these agents as indicative of disturbed pituitary or adrenal function is untenable.

Is the four-hour eosinophil response test to epinephrine or ephedrine a useful clinical tool?

Thorn and Forsham (6) write that ". . . a fall of 50% or more in circulating eosinophils (after injection of epinephrine) excludes the presence of serious pituitary A.C.T.H. or adrenal cortical insufficiency." However, a large number of physicians interpret the test erroneously, and believe that an inadequate response of less than 50% fall indicates disease or relative dysfunction of the hypothalamus, pituitary, or adrenal cortex.

The fact that more than half of our tests with epinephrine or ephedrine in healthy people and in patients showed less than a 50% eosinopenic response attests to the error of such an interpretation. It is obvious that over half of the patients did not have serious dysfunction of the pituitary or adrenal glands. In practically none of the pa-

tients were there other symptoms or findings suggestive of hypopituitarism or neurologic disorders.

We have also shown that neoplastic disease of the pituitary does not necessarily interfere with the eosinopenic response to ephedrine, and Almy, Laragh, and Cohen (35) have noted similar observations. In patients with anorexia nervosa or inanition from other causes we have also recorded large eosinopenic responses to epinephrine and ephedrine. Many observers have postulated that functional hypopituitarism exists in such cases, though others deny this possibility (25). In addition, adrenalectomized patients have responded to epinephrine in the presence of cortisone. Thus, the diagnostic value of negative tests to epinephrine or ephedrine in individual subjects appears to be practically nil. Positive tests to these agents in patients (who are not receiving cortisone) would appear to rule out Addison's disease.

What is the value of these tests in the study of particular diseases or physiologic states? To be certain that the response to an agent such as epinephrine differs significantly from that seen in normals, a control group should be simultaneously tested and the results subjected to statistical analysis. This can be done through use of a "*t*" test or by the graphic method of Figure 5, in which the shaded areas encompassed by the fiducial limits of the respective means are separated and indicate significant differences of response. If these areas had overlapped, one could not say that the differences between the means were significant, and accumulation of further data might or might not indicate significant differences.

Nevertheless, even though an investigator demonstrates significant deviation of a particular group from normal in tests with epinephrine or ephedrine the interpretation of this deviation as differences in adrenal or pituitary function is still open to question. Proper assessment of functional endocrine changes in different groups of subjects must await assay of adrenocorticotropic hormone and/or adrenal steroids in the blood before and after these stimuli.

CONCLUSIONS

Clinical and statistical analysis of 702 four-hour eosinophil response tests to corticotropin, ephedrine, epinephrine, or placebos in 284 normal and miscellaneous medical subjects lead us to conclude:

1. Despite careful technique, individual eosinophil response tests are very inaccurate.

2. Failure of response to corticotropin (A.C.-T.H.) is suggestive of Addison's disease. Repeated four-hour, single dose tests and/or more vigorous attempts at adrenal cortical stimulation should be employed before failure of eosinopenia is attributed to adrenocortical insufficiency.

3. Greater than 50% drop of eosinophils has been noted following epinephrine or ephedrine in patients with pituitary tumors and in patients adrenalectomized and receiving small doses of cortisone. Responses of less than 50% are seen in a large number of patients with unrelated pathological conditions. Tests with these substances are therefore of little value in the diagnosis of adrenal, hypothalamic, or pituitary disease, and do not accurately assess the functional capacity of these organs at the time of examination.

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THE EXCHANGEABLE POTASSIUM CONTENT IN DISEASE STATES [1, 2]

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Although the importance of potassium in physiologic processes has long been recognized, there is a paucity of information concerning its metabolism, particularly in clinical disease states. This scarcity of knowledge has been due primarily to the technical difficulties involved in measurement of soluble cations. The introduction of flame spectrophotometry and the availability of synthetic radioactive isotopes have partially overcome these technical limitations.

Although information is available concerning the external balance of potassium and alterations in the extracellular potassium concentration in various disease states, such data give no direct information concerning the body store of this cation. The injection of radioactive potassium (K^{42}) affords the only direct procedure available for estimating the total potassium content of the body in the intact animal. The specific activity of the urine, after partial equilibration of radioactive potassium with the native atoms, can be used as a measure of the "total exchangeable potassium content" (Ke). Values for normal young males and females have been reported (1, 2). No such studies in individuals with various disease states are yet available.

The purpose of the present study was to determine the extent to which pathologic conditions alter the exchangeable potassium content of hospitalized subjects, and to attempt to correlate alterations in this measurement with the associated metabolic disturbances, and to determine if the administration of "loading" doses of potassium could correct deficiencies detected.

MATERIAL AND METHODS

Subjects

A total of 69 hospitalized subjects—39 males and 30 females—between the ages of 14 and 78 years were studied. The various pathologic states observed are listed in Tables I–IV. All had illnesses of some weeks' duration which justified hospitalization for study and treatment. In addition, six normal individuals, three males and three females, were studied in order to determine the reproducibility of the measurement and the effect of the administration of an oral supplement of potassium.

The subjects were divided into four groups. Group 1 consisted of the normal subjects and the four patients in steady states. Groups 2 and 3, consisting, respectively, of 28 males and 22 females, were used for the original survey study. The individuals in these two groups were selected at random from patients admitted to the medical wards. Studies were performed on any such patient from whom reliable urine collections could be made. Because facilities were limited, it was not possible to include all likely candidates in the study.

Group 4, consisting of nine males and six females, was studied a few months after the study of groups 2 and 3 was completed. In this group serial determinations of Ke were performed in order to study the effect of supplementary potassium chloride. At least two determinations of Ke were performed on each patient in this group.

Plan of the experiment

In the normal subjects in group 1, two determinations of Ke were performed one week apart; the subjects were then given KCl, 3 gm. daily for six days, and the third Ke was measured. The manner in which the other four were studied is described under Comments in Table I.

Single determinations of Ke were done in groups 2 and 3. In group 4, the initial determination of Ke was performed as soon as possible after the patient's admission to the hospital. Upon completion of the initial study, 13 subjects were given oral supplements of potassium chloride, 3 gm. (40 meq.) daily, for a week. Then a second Ke determination was done after which potassium chloride was discontinued. Whenever possible a third determination was performed a week later. Two of the individuals in group 4 were not given supplementary potassium chlo-

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ride; in these cases, the effect of the disease process was observed by serial determinations of Ke.

Experimental procedure

After breakfast, between 8:30 and 9 a.m., each subject was given an intravenous injection of radioactive potassium by calibrated syringe. All urine specimens until 6 a.m. the next morning were collected and pooled, and this collection of urine was measured for the excretion of K^{42}. The specific activity of the urine was determined on two spot samples collected at 7 and 9 a.m. on the day after injection. The subject was asked to empty his bladder completely by voiding. In some instances, a specimen could not be obtained at the exact time, but was obtained as soon thereafter as possible.

No restrictions were placed on activity or on the intake of food, water, or salt. The usual lunch and supper were given on the day of injection. Breakfast the next morning was delayed until after the second spot urine sample was obtained.

Preparation and administration of radioactive potassium

All shipments of K^{42} were treated in the manner described by Corsa and his co-workers (1). Usable shipments were usually received early Tuesday morning, and most of the injections were made later that morning. Approximately 1.5 meq. of potassium chloride solution, containing 100 microcuries of K^{42}, were injected intravenously. A few injections were given on Wednesday, and these patients received 3 meq. of potassium chloride solution containing 50 microcuries of K^{42}.

Measurement of radioactivity

The activity of the urine specimens was determined with a dipping tube and a scaling circuit. Counts were made to 1% accuracy. All counting rates were at least ten times background, and were usually in the range of 500 to 3,000 per minute. At this range of counting rate, no dead time correction for the Geiger tube was necessary. All determinations were corrected for decay.

Preliminary studies confirmed the observations of Corsa and his associates (1) that the specific activity of potassium in the urine reached an equilibrium by 24 hours. The mean difference in specific activity between the two spot specimens, when expressed as per cent of the mean Ke, was $6.52 \pm 6.65\%$.

TABLE I

Reproducibility of exchangeable potassium measurements and the effect of oral supplements of potassium in normal subjects and in patients in steady states

Subjects	Ke_1		Ke_2		Ke_3	
	(meq.)	(meq./Kg.)	(meq.)	(meq./Kg.)	(meq.)	(meq./Kg.)
Normal Subjects						
Women						
1	2,500	45.9	2,219	40.7	2,280	38.2*
2	1,947	39.3	1,682	34.0	1,679	33.9*
3	2,160	38.9	2,155	38.8	2,358	42.5*
Men						
1	3,641	43.3	3,447	41.0	4,145	49.3*
2	3,537	48.1	3,261	44.3	3,753	51.0*
3	4,064	38.1	4,007	37.5	4,136	38.7*

Patients in Steady States

Patient	Age, sex	Diagnosis	Ke_1		Ke_2		Ke_3		Comments
			(meq.)	(meq./Kg.)	(meq.)	(meq./Kg.)	(meq.)	(meq./Kg.)	
Ba	M 71	Diabetes with peripheral vascular disease	1,544	25.3	1,524	24.9	1,252	20.6*	Diabetes well regulated Supracondylar amputation between Ke_2 and Ke_3
Ho	M 72	Diabetes with peripheral vascular disease	1,848	34.7	1,750	32.9*	1,894	36.6	Diabetes well regulated
Bo	F 59	Myxedema and diabetes	1,553	21.4	1,576	21.7			Myxedema for several years. Diabetes well regulated. No potassium given
Th	F 38	Ulcerative colitis	1,641	34.7	1,638	34.6	1,811	37.6	Duration 8 years. In relapse during study. Interval of 2 wks. between Ke_2 and Ke_3

All Ke determinations performed at weekly intervals unless otherwise noted
*Ke determination after administration of oral supplement of KCl, 3 gm. d. .ly, for six days.

TABLE II

Exchangeable potassium content in males with disease states (group 2)

Patient	Age (yrs.)	Diagnosis	Ke (meq.)	Body weight (Kg.)	Ke/Wt. (meq./Kg.)	Remarks
1	32	Hypertensive cardiovascular disease	3,797	64.5	58.9	Duration 11 yrs. Symptomatic for 6 mos. Clinically well nourished.
2	51	Neurosyphilis	4,016	68.3	58.8	Spinal fluid protein = 150 mg. %. Clinically well nourished.
3	48	Polycystic kidneys with azotemia	3,289	60.8	54.1	Hypertension for 3 yrs. NPN = 166 mg. %. No generalized edema.
4	42	Ulcerative colitis	2,767	53.6	51.6	Duration 6 mos. 34 lbs. weight loss.
5	66	Pneumoconiosis and pulmonary emphysema	2,772	54.1	51.2	Arteriosclerotic heart disease. Slender.
6	37	Pyelonephritis	3,097	61.4	50.4	Minimal symptoms for 6 mos. No recent weight loss or generalized edema. NPN = 65 mg. %.
7	56	Iron-deficiency anemia	2,942	60.0	49.0	Duration 6 mos. Weight loss of 15 lbs.
8	49	Duodenal ulcer, adenocarcinoma of cecum	2,608	54.5	47.9	Clinically emaciated. Weight loss of 20 lbs. in 5 mos. No anemia or hypoproteinemia.
9	25	Convulsive disorder, pyrexia of undetermined origin	2,679	57.5	46.6	Symptomatic 2 wks. Afebrile at time of determination.
10	71	Generalized arteriosclerosis	2,629	56.6	46.4	Chronic blood loss from internal hemorrhoids. Hemoglobin = 10.5 gm.
11	58	Diabetes mellitus	2,088	45.8	45.6	Duration 3 yrs. Poorly controlled 8 mos., with 20 lbs. weight loss.
12	19	Peptic ulcer	2,912	65.1	44.7	Epigastric symptoms for 18 mos. Hematemesis 8 days prior to admission.
13	14	Congenital heart disease	1,358	30.5	44.5	Interventricular septal defect. Cryptorchidism.
14	16	Trichinosis, peptic ulcer	2,478	55.9	44.3	Duration 2.5 wks. Eosinophilia (25%). Positive trichinella skin test.
15	40	Cerebral aneurysm	3,282	79.1	41.5	Symptoms limited to complaint of headaches. Normal otherwise.
16	56	Neurosyphilis	2,370	57.7	41.1	Spinal fluid protein = 102 mg. %.
17	49	Diabetes mellitus	3,383	86.8	39.0	Well regulated. Admitted for therapy of acute upper respiratory infection.
18	58	Chronic cholecystitis with cholelithiasis	2,801	73.0	38.4	Faint icterus. Clinically well nourished.
19	56	Rheumatic and arteriosclerotic heart disease	2,535	69.3	36.6	Digitalized; no edema or weight loss. Auricular fibrillation.
20	41	Progressive peroneal atrophy	1,562	43.4	36.0	Duration 30 yrs.
21	57	Portal cirrhosis	2,053	61.3	33.5	Neurodermatitis disseminata for 8 mos. 28 lbs. weight loss in 2 mos. 45% BSP retention.
22	76	Arteriosclerotic heart disease	2,500	75.2	33.3	Progressive congestive heart failure 14 mos. 20 lbs. weight loss in 1 yr. Pitting edema.
23	78	Adenocarcinoma of head of pancreas	1,329	42.3	31.4	30 lbs. weight loss in 1 mo. prior to admission. Known weight loss for 1 yr. Anorexic.
24	69	Thyrotoxicosis, arteriosclerotic heart disease	1,413	45.5	31.1	Parkinsonism 35 yrs. Duration of thyrotoxicosis, 8 mos. In relapse.
25	56	Arteriosclerotic heart disease, pulmonary embolism	1,437	48.0	29.9	Duration heart disease 15 mos. with 40 lbs. weight loss. Loss of 20 lbs. in 10 days on mercurials and digitalis.
26	42	Metastatic carcinoma	2,350	82.7	28.4	Weight loss 20 lbs. in preceding 4 mos. Persistent fever. Primary site in colon.
27	55	Malnutrition, empyema, pneumothorax	864	32.5	26.6	Hypertensive cardiovascular disease. 75 lbs. weight loss. Expired 10 days after determination.
28	16	Inactive rheumatic heart disease	1,667	67.7	24.6	Acute rheumatic fever 3 yrs. previously. Normal sedimentation rate at time of determination.

Chemical determinations

Total *potassium concentration* in the urine was determined by flame spectrophotometry, using the method of Mosher and his associates (3).

Calculation of exchangeable potassium

The following formula was used to calculate the value for the exchangeable potassium content of the body:

$$Ke = \frac{K_i^{42} - K_u^{42}}{\frac{Ku^{42}}{Ku^{39}}}$$

Ke = quantity of exchangeable potassium in milliequivalents.

K_i^{42} = quantity of radiopotassium administered (arbitrary units).

K_u^{42} = quantity of radiopotassium excreted in the urine within 21 to 21.5 hours after its intravenous injection.

Ku^{42} = concentration of radiopotassium in the urine samples.

Ku^{42}/Ku^{39} = mean specific activity of two spot specimens.

RESULTS

Group 1 (Table I)

Better reproducibility of Ke was obtained in patients in steady states than in normal young subjects. However, the activity and the diet of the individuals during the study was better controlled in the patients. The mean difference between the two Ke determinations made a week apart without oral supplementation was 125 meq. or 2.0 meq./Kg. Following the administration of an oral supplement, the second Ke was higher by a mean of 212 meq. or 2.2 meq./Kg. In the interpretation of the results in the other groups, an increase of greater than 250 meq. or 5 meq./Kg. was considered of significance, although it is rec-

TABLE III

Exchangeable potassium content in females with disease states (group 3)

Patient	Age (yrs.)	Diagnosis	Ke (meq.)	Body weight (Kg.)	Ke/Wt. (meq./Kg.)	Remarks
1	25	Diabetes mellitus	2,201	43.6	50.5	Duration 9 yrs. Poor control. Urinary sugar 3 + on day of determination.
2	55	Diabetes mellitus	2,540	55.9	45.5	Clinically well nourished. Duration 4 yrs. with poor control. Neurogenic bladder.
3	34	Chronic anxiety state	1,811	45.2	40.1	Duration 2 yrs. Weight loss 20 lbs.
4	54	Vascular lesion, basal ganglion	1,805	46.4	38.9	Tremor of right arm, progressive for 3 yrs. Recent involvement of right leg.
5	67	Gastric ulcer	1,368	36.0	38.0	Very slender. Quiescent bronchial asthma.
6	42	Hypertensive cardiovascular disease	1,789	47.5	37.7	No history of weight loss.
7	59	Hypertensive and arteriosclerotic heart disease	2,230	60.9	36.6	Pulmonary fibrosis. Digitoxin, 0.8 mg., given during determination.
8	52	Psychoneurosis	1,498	43.2	34.7	Weight loss and diarrhea for several months. Narcotic addiction.
9	21	Ulcerative colitis, emetine intoxication	1,551	44.8	34.6	Duration colitis, 7 yrs. Emetine therapy 1 mo. previously; subsequently developed generalized weakness and tachycardia.
10	43	Duodenitis, anemia	1,666	51.8	32.2	Iron-deficiency anemia. Asymptomatic intestinal amebiasis.
11	41	Rheumatic heart disease	1,726	54.1	31.9	Mitral stenosis and insufficiency. On digitalis and quinidine.
12	54	Rheumatoid arthritis	1,609	55.9	28.8	Progressive disease for 2 yrs. Bronchial asthma for 30 yrs.
13	30	Exogenous obesity	2,700	95.2	28.4	Typical fat distribution.
14	55	Osteoarthritis	2,031	71.6	28.4	Slightly obese.
15	21	Chronic anxiety state	1,439	50.9	28.3	Emotional immaturity. Clinically simulated thyrotoxicosis. Weight loss of 7 lbs. in 3 wks. before admission.
16	24	Multiple sclerosis	1,380	49.5	27.9	In relapse. Duration of illness, 1 yr. No recent weight loss.
17	65	Generalized urticaria	1,435	53.2	27.0	Urticaria for 2.5 mos., etiology unknown. Postmenopausal osteoporosis.
18	73	Rheumatoid arthritis and cerebrovascular accident	1,221	47.3	25.8	Chronic disease state. Arthritis for several years. Generalized arteriosclerosis.
19	44	Obesity, anemia	1,675	67.5	24.8	Iron-deficiency type. Anticoagulant therapy for retinal vein thrombosis.
20	19	Diabetes mellitus	1,673	72.0	23.2	Duration 2 yrs. Urinary sugar 4+.
21	44	Psychoneurosis	1,307	64.5	20.3	Tension headaches, psychic anorexia, nausea and vomiting. Weight loss of 30 lbs.
	45	Exogenous obesity, hypertensive cardiovascular disease	1,281	68.2	18.8	Mild cerebrovascular accident.

ognized that the retention of administered potassium over a short period may not be definite evidence for a prior depletion.

Group 2 (Table II)

When the values for Ke/Wt. from this group of male subjects with various disease states were compared with those found in 30 normal young males (in whom the range was 35.6 – 53.6 meq./Kg.), three subjects (Cases 1, 2, and 3) had values higher than those found in the normal males. Eight of the 28 subjects had values lower than 35.6 meq./Kg. The disease states in which these low values were found included inactive rheumatic

TABLE IV

Exchangeable potassium content in subjects studied serially (group 4)

Patient, sex	Age (yrs.)	Diagnosis	Ke (meq.)	Body weight (Kg.)	Ke/Wt. (meq./Kg.)	Remarks
Group 4A						
1 M	39	Carcinoma metastatic to the liver	1,566*	55.0	28.5	Onset with weakness and weight loss of 20 lbs. in 5 mos. Confirmed by liver biopsy.
			1,600†	51.0	31.4	
2 F	65	Myxedema	1,586*	75.0	21.1	Duration 8 yrs. Refractory anemia. Inadequate protein intake. Thyroid therapy started 3 days after Ke.*
			1,366†	72.7	18.8	
			1,638‡	72.8	22.5	
3 F	65	Myxedema	1,679§	62.4	27.9	Classic symptoms and signs. Anemia for 4 yrs. Hepatomegaly and ascites. Liver biopsy showed fatty metamorphosis. No supplementary K given.
			1,497	60.9	24.6	
4 M		Fever of unknown origin	1,814§	47.7	38.0	Duration of 30 days. No known weight loss. Febrile during studies.
			1,643	47.7	34.4	
5 F	46	Partial intestinal obstruction	1,235*	45.5	27.1	Due to postoperative adhesions. Symptoms suggestive of peptic ulcer for 8 mos. 10 lbs. loss in 4 mos. Diagnosis confirmed at surgery.
			1,344†	45.5	29.5	
6 F	18	Rheumatic heart disease, active	1,014*	45.9	22.1	Recurrent rheumatic activity for 7 yrs. with valvular heart disease, low grade fever and elevated sedimentation rate.
			941†	43.6	21.6	
Group 4B						
1 M	55	Cirrhosis	2,084*	75.5	27.7	History of alcoholism for 30 yrs. with inadequate diet. Ankle edema, ascites for 9 wks. before Ke.*
			2,550†	68.6	37.2	
			2,423‡	68.2	35.5	
2 M	39	Rheumatic heart disease	1,980*	62.8	31.5	Mitral stenosis and insufficiency, aortic insufficiency. Duration at least 10 yrs. On digitalis.
			2,063†	58.9	35.0	
			2,315‡	59.4	39.0	
3 F	24	Multiple sclerosis	1,380*	49.5	27.9	Duration 3 yrs. Studied during a relapse.
			1,657†	49.5	33.5	
4 M	41	Psychoneurosis, adenoma of thyroid	2,764*	83.2	33.2	Vague complaints. Weakness prominent.
			3,135†	83.2	37.7	
			2,967‡	83.2	35.7	
5 M		Chronic glomerulonephritis	2,555*	79.5	32.1	Duration symptoms 11 yrs. Hypertension 3 yrs. Worse past 3 mos. PSP = 9% in 1 hr. NPN = 87 mg. % with Ke.* NPN increased to 154 mg. % on day of Ke.‡
			3,263†	77.3	42.2	
			2,505‡	74.1	33.8	
6 M		Diabetes mellitus	1,553*	52.5	29.6	Duration of diabetes 5 yrs. with poor regulation. Peptic ulcer symptoms for 2 mos. Developed pyloric obstruction and was placed on nasogastric suction between Ke† and Ke.‡ Metabolic alkalosis with Ke.‡
			1,873†	48.3	38.8	
			1,463‡	47.5	30.8	
Group 4C						
1 F	36	Chronic pulmonary fibrosis	2,928*	52.6	55.7	Chronic bronchitis progressive for 13 yrs., with fibrosis. Prone to hyperventilate. 36 lbs. weight loss in 2 yrs.
			2,157†	52.6	41.0	
			2,425‡	52.6	46.1	
2 M	52	Pneumoconiosis, gastric ulcer	3,472*	57.7	60.2	Progressive cough and dyspnea for 16 yrs. with roentgenologic changes. Diagnosis of ulcer established while hospitalized.
			2,657†	57.7	46.0	
			1,776‡	57.7	30.1	
3 M	41	Ulcerative colitis	3,113*	68.2	45.6	Duration 1 mo. 14 stools daily. 40 lbs. weight loss in 6 mos. Asymptomatic peptic ulcer. With Ke*, NPN = 76 mg. %, with Ke†, NPN = 43 mg. %.
			2,319†	68.8	33.8	
			1,916‡	66.8	28.7	

Ke determinations were made at weekly intervals unless otherwise indicated.

* Ke before the administration of KCl.

† Ke after one week of KCl, 3 gm. daily.

‡ Ke one week after discontinuation of KCl.

§ Not given supplementary KCl.

heart disease, malnutrition, carcinoma, arteriosclerotic heart disease, thyrotoxicosis, and cirrhosis.

Group 3 (Table III)

The range of Ke/Wt. in 20 normal young adult females was previously found to be 25.1 to 35.9 meq./Kg. Of the 22 individuals in group 2, seven subjects had values higher than those found in the normal group. Four of the 22 had values below 25.1 meq./Kg. The diagnoses in these four subjects were, respectively: diabetes mellitus, psychoneurosis, obesity and anemia, and exogenous obesity with hypertensive cardiovascular disease.

Group 4 (Table IV)

The results of the study in this group have been divided into three categories on the basis of the type of response to supplementary potassium chloride. Group 4A consists of six individuals who did not show what was considered to be a significant change (± 250 meq.) in Ke when placed on supplementary oral potassium chloride. Group 4B consists of six individuals who showed an increase in Ke at the second or third determination, or with both measurements. Group 4C consists of three individuals in whom the Ke value decreased during the administration of potassium supplements.

Group 4A. No change in the Ke occurred following the administration of potassium supplements in subjects with metastatic carcinoma, myxedema, fever of unknown origin, partial intestinal obstruction, and rheumatic heart disease.

Group 4B. The supplementary feeding of potassium chloride produced an increase in Ke of more than 250 meq. in the following disease states: cirrhosis, rheumatic heart disease, psychoneurosis, multiple sclerosis, chronic glomerulonephritis, and poorly regulated diabetes mellitus.

Group 4C. In these three instances, Ke decreased during the administration of supplementary potassium: chronic pulmonary fibrosis, pneumoconiosis and gastric ulcer, and ulcerative colitis.

COMMENT

It has been recognized in the past that in a few disease states certain abnormalities in potassium metabolism, as evidenced by alterations in the external balance or concentration in the serum, are associated with clinical symptoms and signs. A body deficiency of potassium is known to exist in diabetic acidosis and coma (4), in certain gastrointestinal disorders (5), and in certain postoperative conditions (6). With severe renal damage, retention of potassium may occur. The external balance method, however, gives no direct evidence of any imbalance in the distribution of potassium between the intra- and extracellular fluid compartments, although such an imbalance has been suspected. The difficulty of confirming clinically the suspicion of an intracellular potassium deficiency has been recognized previously. The finding of a low serum potassium concentration is highly suggestive of the presence of body deficiency. However, it is important to realize that a total body deficiency may exist in the presence of a normal serum concentration. All of the serum potassium determinations done on the patients in the present study were within the range of 3.5 to 5.0 meq./Kg.

The availability of radioactive isotopes has provided a method for measuring directly the body content of potassium. Since more than 95% of the body's store of potassium is normally situated within cells, such a measurement of the total body content of potassium should serve as a relatively reliable index to the intracellular store of this cation.

Previous determinations of the exchangeable potassium content in normal male and female subjects have established presumably normal ranges (1, 2). Although it is recognized that such studies to date have been limited to fairly homogeneous groups of young subjects, these values are the only ones available at present for comparison with Ke values obtained in disease states.

The finding, in some diseased males and females, of Ke/Wt. values higher than those obtained in normal subjects suggests that the former group was more muscular than normal subjects. Such a finding suggests that the loss of body fat may elevate the relative fraction of lean tissue. Except in the case of the individual with chronic glomerulonephritis, it is difficult to explain the higher values for Ke/Wt. on the basis of a pathologic state. In severe renal failure, retention of potassium may occur when the intake exceeds the excretory capacity of the kidneys.

The results of the present study seem to indicate that several factors may produce a low Ke/Wt. value:

1. A decrease in body mass secondary to (*a*) a decrease in food intake resulting from anorexia, (*b*) an increase in the catabolic processes, or (*c*) both.

The loss in total tissue mass, in such an event, would parallel the loss of body weight. The concentration of potassium in the remaining tissues, however, would be unchanged, so that no body deficiency of potassium would result. This factor is probably the one principally responsible for the low Ke/Wt. values in the individuals with malnutrition, malignancy, thyrotoxicosis, and chronic infectious diseases or febrile episodes. It has been shown experimentally that during starvation normal rabbits lose body potassium progressively with loss of body weight, but that the concentration of this cation in the remaining tissues is unaltered (*7*).

2. An increase in the relative fat content of the body, such as occurs with exogenous obesity (Table III, Cases 19 and *22*).

3. Expansion of the extracellular fluid compartment as a result of edema or the deposition of myxedema fluid.

Should the low values for Ke/Wt. be due to any or all of the above causes, no decrease in the intracellular concentration of potassium occurs; therefore, no increase in the potassium content of the body would be expected when supplementary potassium is administered. Such was the result obtained in the cases of carcinoma, myxedema, chronic infectious disease, fever of unknown origin, and untreated rheumatic heart disease.

4. Excessive loss of potassium from the body, usually by the gastrointestinal tract, as the result of vomiting, diarrhea, or intestinal suction.

The potassium concentration of the intestinal secretions is several times higher than that of serum. With loss of intestinal contents, a decrease in the intracellular concentration of potassium results. Such a deficit might be corrected simply by supplying potassium orally or parenterally.

5. A cellular metabolic abnormality, which may lead to an intracellular deficiency.

A low value for Ke/Wt. may lead one to suspect such an abnormality. An increase in this value following the administration of supplementary potassium, particularly if such an increase is maintained after the supplement is discontinued, is certainly suggestive of a prior deficiency state. Such an intracellular potassium deficiency appears to be present in poorly regulated diabetes mellitus, and has already been shown to exist in diabetic acidosis and coma. A potassium deficit might be anticipated in cirrhotic patients because of the impairment in carbohydrate metabolism.

In the patients with psychoneurosis and multiple sclerosis, the increase in the Ke values following the administration of potassium supplement suggests the existence of an occult potassium deficiency in these states. Further studies are required to confirm these findings and to explain the mechanism.

SUMMARY

The use of a radioactive isotope of potassium, K^{42}, has made possible the direct measurement of the exchangeable potassium content of the body. Such a determination was performed in 69 hospitalized subjects with various diseases. With few exceptions, the values obtained were either within the range found in normal young subjects, or lower. Although some of these low values could be attributed to the loss of body tissue resulting from chronic illness, the low values in certain disease states suggested the presence of a body deficiency of the potassium ion.

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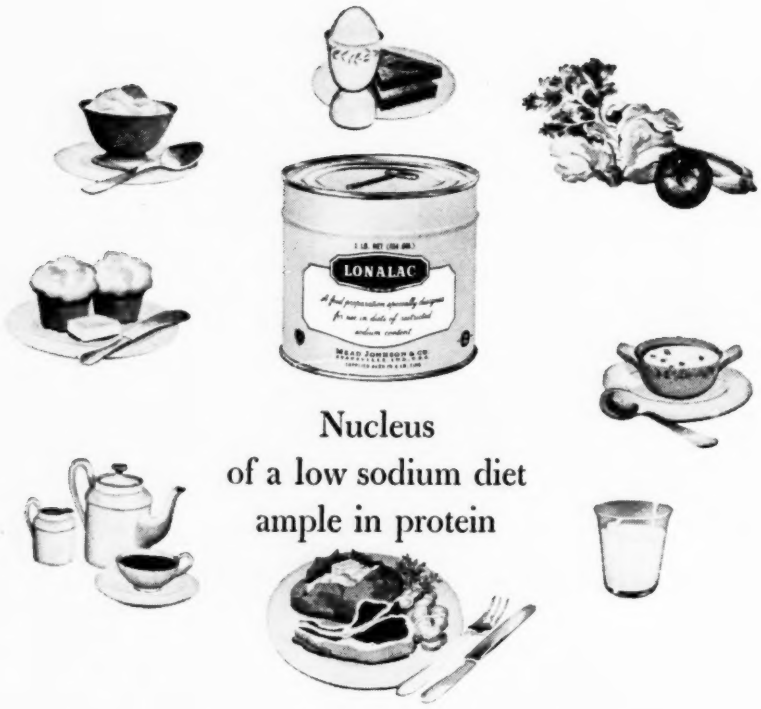

Nucleus of a low sodium diet ample in protein

With the same generous protein content as whole milk powder, Lonalac contains only one fiftieth of the sodium found in milk.

Low sodium diets, which are often indicated in the management of congestive heart failure, hypertension, obesity in children and other clinical conditions, find valuable implementation in LONALAC.®

Whereas most foods abundant in high quality protein contain large quantities of sodium also, Lonalac has a negligible 0.02%.

Nutritionally similar to whole milk, Lonalac can be used in any way in which milk is used—as a beverage, as a replacement for cream, or in soups, white sauces, breads, cakes, muffins, puddings and other appetizing dishes.

Used to replace milk, Lonalac can be combined with limited amounts of meat and eggs, and with low sodium foods, to provide nutritionally adequate diets containing as little as 200 mg. of sodium daily.

Flexible low sodium diet outlines and recipes employing Lonalac are available on request.

LONALAC is supplied in 1 and 4 pound tins.

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